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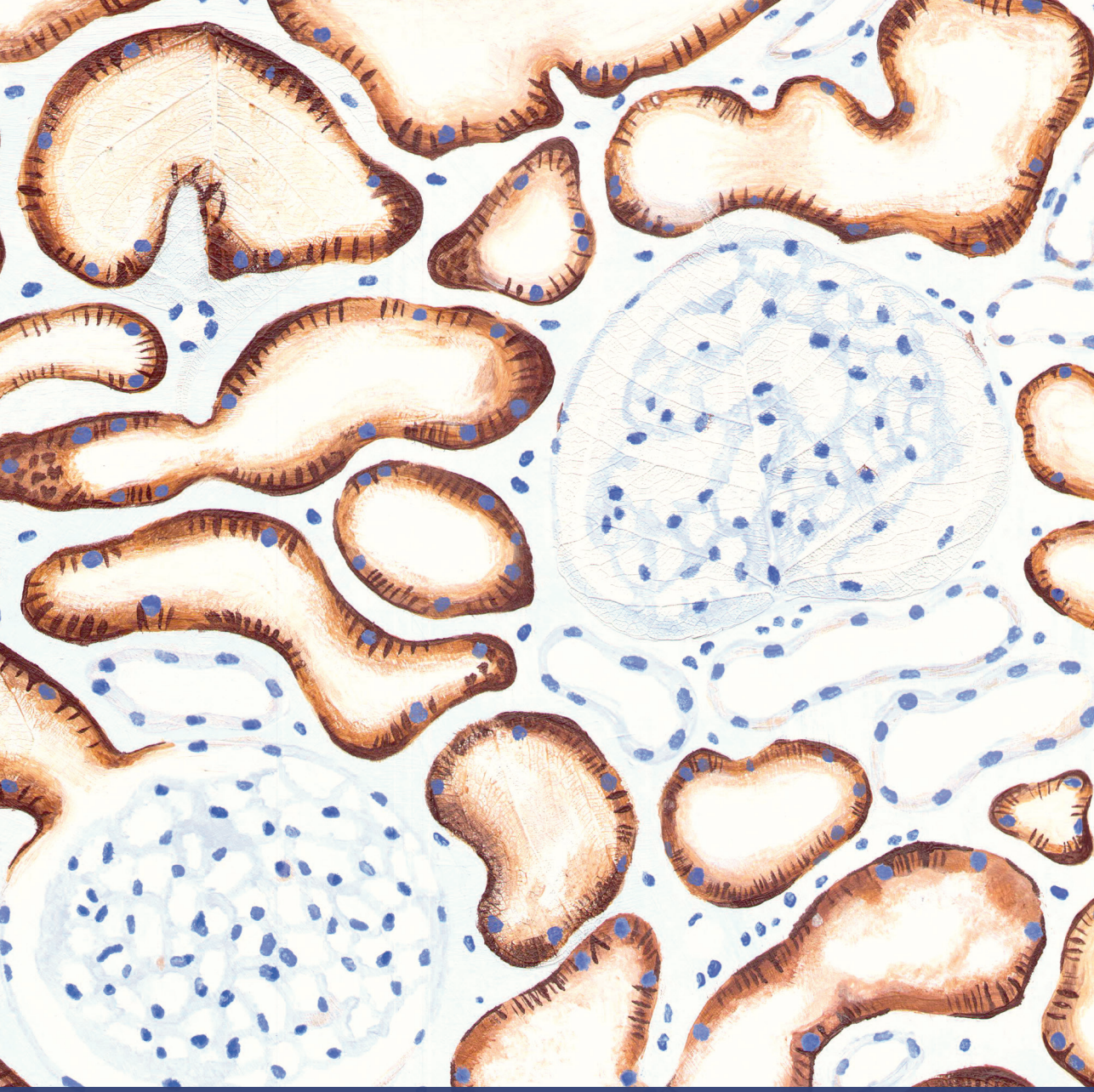

Coming into view:

Renal iron handling in human health and disease

Sanne van Raaij

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The research presented in this thesis was performed at the Translational Metabolic Laboratory, part of department of Laboratory Medicine, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, the Netherlands. Publication of this thesis was financially supported by the Radboud University Nijmegen, the Netherlands.

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Over markers in eiwitten

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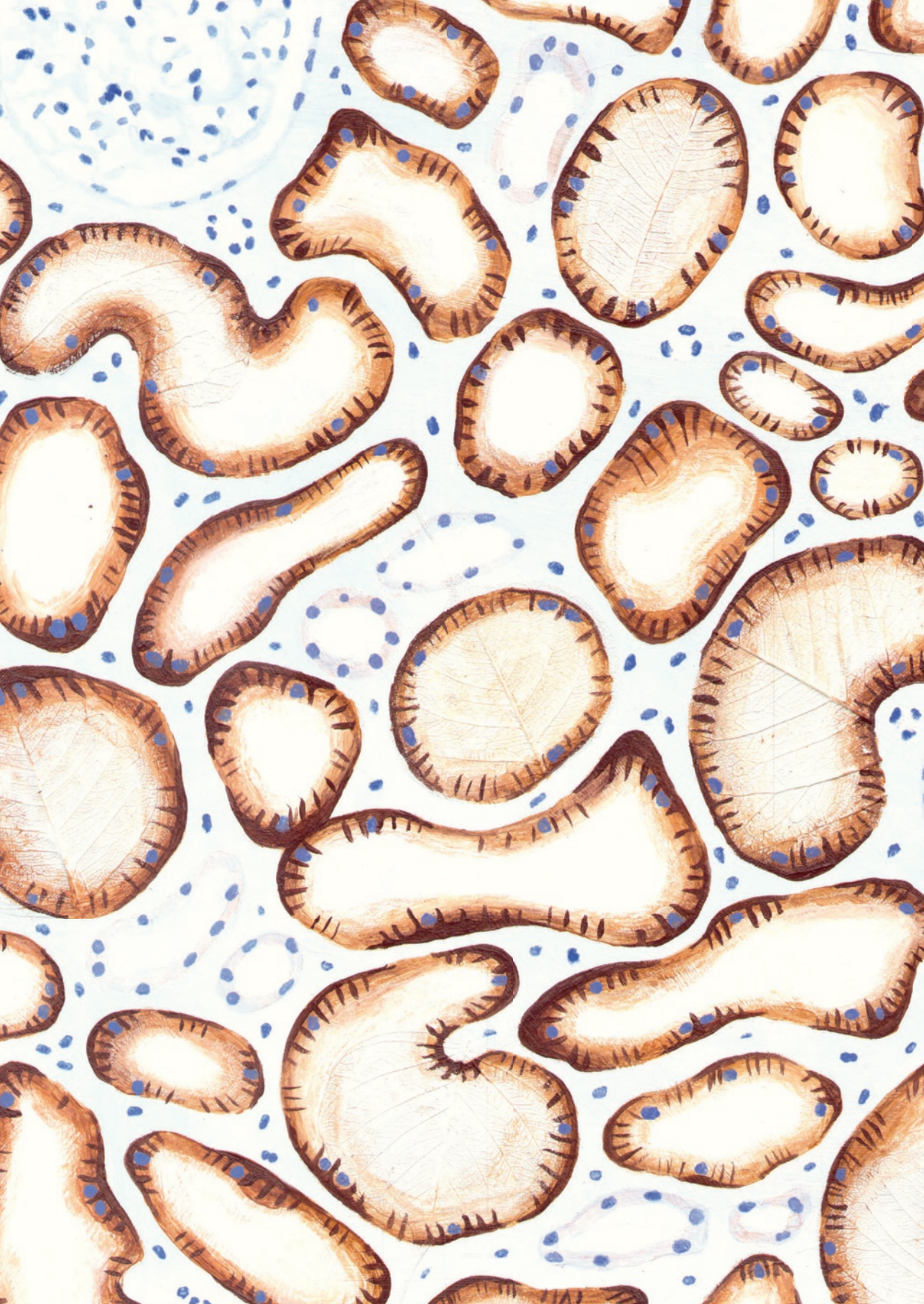

1

General introduction

IRON HOMEOSTASIS

The metal iron

Iron is the most abundant metal on earth.[1] Iron itself is insoluble in water and, therefore, it needs to be complexed to ligands to become bioavailable to living organisms.[1] In the mammalian systemic circulation, iron is incorporated in hemoglobin in erythrocytes to transport oxygen throughout the body.[2] At the cellular level, iron is incorporated in various enzymes and functions in a large variety of cell processes ranging from cell proliferation and the mitochondrial respiratory chain, to cellular electron transport and DNA synthesis.[3]

Iron functions in many processes via its reversible shifting between two redox states: the oxidized ferric iron (Fe^{3+}) and the reduced ferrous iron (Fe^{2+}).[4] Most iron in the body is in the ferric form, since physiological oxygen concentrations readily oxidize ferrous iron to ferric iron. As a result, reducing agents are critical, to provide ferrous iron that is required for cellular transport, storage and heme synthesis.[4] These redox shuttling properties can also be harmful. In presence of oxygen, iron redox cycling is prone to catalyze the production of highly reactive oxygen species (ROS), generated in the Fenton and Haber-Weiss reactions (Figure 1.1).[5,6] Iron-induced ROS, particularly hydroxyl radicals,[7] are known to cause injury to cellular structures including membranes, DNA and proteins.[8] Therefore, iron levels are carefully balanced and tightly regulated at both the systemic and cellular level.[9]

Systemic iron homeostasis

The human body contains 3-4 g of iron, the majority of which is incorporated in the form of hemoglobin in circulating erythrocytes or in immature erythrocytes residing in the bone marrow (1-2g) (Figure 1.2).[2] Tissues such as the liver and muscle also contain relatively high iron levels (~1000 mg, and ~300 mg, respectively). In contrast, plasma iron levels are rather low, only 2-3 mg. In the circulation, iron is bound to the glycoprotein transferrin (transferrin-bound iron, TBI), which binds two molecules of iron with a very high affinity (K_d = ~10^{-23} M).[10] In the form of TBI, iron is transported to all cells of the body. Plasma iron levels are predominantly maintained by erythrocyte turnover in reticuloendothelial macrophages in the spleen and replenished by iron absorption from the diet by duodenal enterocytes.[11] Iron transport towards the plasma is mainly controlled by hepcidin. This iron-regulatory peptide hormone is produced in hepatocytes and secreted into the blood stream where it functions via inactivation of ferroportin (SLC40A1), which is the only known mammalian cellular iron exporter. Binding of hepcidin to ferroportin at the plasma membrane causes ferroportin internalization and lysosomal degradation.[12] This way, hepcidin controls dietary, stored and recycled iron transport into the blood stream in

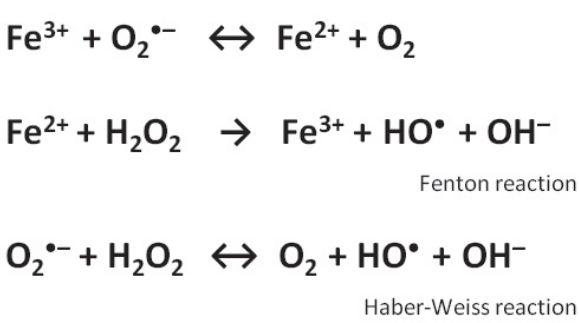

Figure 1.1: Iron can induce reactive oxygen species formation in the Fenton and Haber-Weiss reaction

enterocytes, hepatocytes and macrophages, respectively.[2] As a result, in case of low plasma iron, decreased hepcidin production enables the elevation of circulating iron levels to meet erythropoiesis demand, whereas high hepcidin levels halt iron export into the blood stream when systemic iron levels are high. However, once iron is reabsorbed, the body has limited physiological mechanisms to regulate iron excretion.[9] Daily iron loss is minimal, only 1-2 mg/day, in order to ensure availability of this scarce metal for body iron demand. As a consequence, duodenal enterocytes absorb only 1-2 mg of iron/day. However, iron loss is not actively regulated, and takes place via uncontrolled processes like sloughing of enterocytes, desquamation of skin and urinary cells, bleeding (including menstruation) and sweating.[13,14] Consequently, in physiological conditions, mammalian total body iron levels are mainly regulated through dietary iron uptake at the level of the intestine.[11]

Cellular iron homeostasis

Mammalian cells have evolved strategies to safely acquire, distribute, store and use iron species (Figure 1.3). Most cell types acquire iron via transferrin receptor 1 (TfR1) at the cell surface. At physiological pH (7.4), TfR1 effectively binds diferric transferrin (holotransferrin) and ferric transferrin (monotransferrin), whereas the affinity of iron deficient transferrin form (apotransferrin) for TfR1 is two magnitudes lower.[15] The TBI-TfR1-complex is internalized into an endosome, where acidification via a V-ATPase proton pump lowers the pH to ~5.6, and iron is released from the holotransferrin-TfR1 complex.[15,16] The apotransferrin-TfR1 complex is returned to the cell surface, where the physiological pH now enables dissociation of apotransferrin from its receptor.[15] In the endosome, iron is reduced by a reductase, for example STEAP3 (six-transmembrane epithelial antigen of prostate 3) in erythrocyte precursors,[17] and transported over the endosomal membrane into the cytosol by a divalent metal transporter. Divalent metal transporter 1 (DMT1, SLC11A2) has been reported to transport iron into the cytosol in erythroid precursors, hepatocytes and macrophages.[18-21] However, cellular iron import

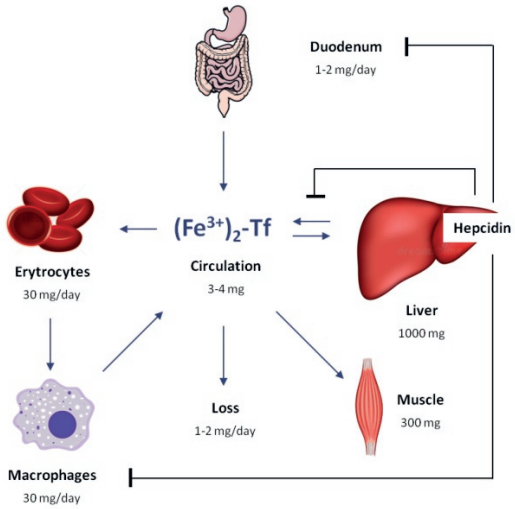

Figure 1.2 Iron uptake, distribution and recycling to maintain sufficient systemic iron levels
The amount of iron bound to transferrin (Tf) in the systemic circulation is maintained by iron absorption in the duodenum to compensate for daily iron loss. Most body iron is used for production of hemoglobin in erythrocytes. Senescent erythrocytes are recycled by reticuloendothelial macrophages, replenishing plasma iron levels. Moreover, iron is stored in hepatocytes (liver), muscle tissue and reticuloendothelial macrophages. Systemic iron homeostasis is regulated by the peptide hormone hepcidin that is produced by hepatocytes when body iron levels are high, causing iron to be sequestered in macrophages, and duodenal iron absorption to be reduced.

redundancy was observed for DMT1, since DMT1 knockout mice become anemic, but maintain liver iron stores.[18] Moreover, divalent metal transporters ZIP8 (SLC39A8) and ZIP14 (SLC39A14) are candidates for endosomal iron transport towards the cytosol in hepatocytes and neuronal cells.[22-24] In the cytosol, iron is either stored in ferritin, utilized by iron-requiring processes, like respiration in the mitochondria, or exported into the blood stream by ferroportin.[25] Ferritin is a large intracellular iron storage protein, where iron is oxidized by the ferroxidase H-ferritin for storage as ferrihydrite aggregates within the L-ferritin core.[26] How iron is transported throughout the cell, *i.e.* from the endosomes to the ferritin molecules or towards ferroportin, is not well known. These transport processes are suggested to be mediated by iron chaperones, like poly(rC) binding proteins (PCBPs), monothiol glutaredoxin (Grx) proteins or cytosolic molecules like glutathione.[27,28]

The above described cellular iron transport mechanisms are tightly regulated to balance cellular iron homeostasis without potential harmful effects. Posttranscriptional iron-responsive element – iron responsive protein (IRE-IRP) regulation has emerged as a central coordinating system to regulate the expression of iron uptake, storage and export proteins.[29] Depending on the cellular iron content, IRP proteins 1 and 2 (IRP1 and IRP2, respectively) bind to IRE mRNA motifs and increase or decrease protein translation, depending on the location of the IRE in the mRNA. Binding of IRPs to single IREs in the

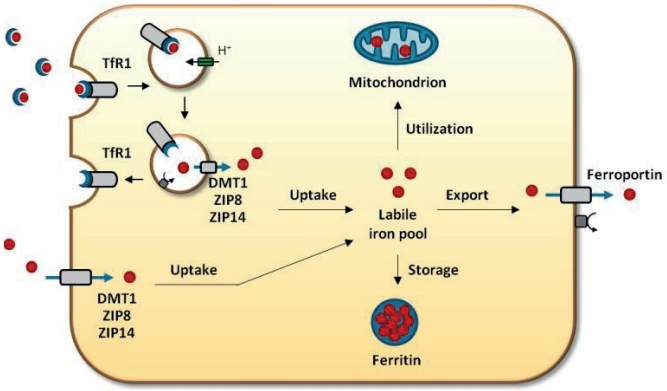

Figure 1.3 Cellular iron handling

Overview of cellular iron handling based on processes described in hepatocytes, enterocytes and macrophages. Iron (in red) bound to transferrin (in blue) (transferrin-bound iron, TBI) is internalized by endocytic transporter transferrin receptor 1 (TfR1). In the endosome, acidification by a V-ATPase proton pump results in release of iron from the TBI-TfR1 complex, which is reduced by a ferrireductase and subsequently transported into the cytosol by potential divalent metal transporters ZIP8, ZIP14 and divalent metal transporter 1 (DMT1). The remaining transferrin-TfR1 receptor complex is returned to the cell surface. Iron bound to small ligands (non-transferrin-bound iron) can potentially be internalized into the cytosol by divalent metal transporters ZIP8, ZIP14 and DMT1. Once in the intracellular labile iron pool, iron can be either utilized in iron-requiring processes in mitochondria, stored in the ferritin protein shell or exported through the cellular iron exporter ferroportin.

5'UTR, for example in L-ferritin, H-ferritin and ferroportin, represses mRNA translation. In contrast, binding of IRPs to multiple IREs located in the 3'UTR in TfR1 and DMT1 results in mRNA stabilization and, as such, inhibits mRNA decay and enhance mRNA translation.[30] In case of low intracellular iron levels, IRPs can freely bind IRE mRNA elements. This results in decreased iron export by ferroportin and reduced iron storage in ferritin, and iron is released from ferritin via NCOA4-mediated autolysosomal ferritin degradation.[31,32] Moreover, TfR1 translation is prolonged, further increasing intracellular iron levels.[29] In contrast, with high intracellular iron levels, iron is incorporated in IRP1 to form an iron sulfur cluster that functions as an intracellular aconitase in the Krebs cycle with the assistance of FAM96A, whereas iron binding in IRP2 results in proteosomal degradation mediated by FBXL5.[33,34] This leaves the IREs unbound, resulting in L-ferritin, H-ferritin and ferroportin translation, increasing iron storage and iron export, while TfR1 mRNA are no longer stabilized and will be degraded, decreasing cellular iron uptake.[29] All together, this decreases intracellular iron levels. To date, IRE-IRP regulation of DMT1 is not fully resolved, as this seems life-stage dependent[35] and some DMT1 isoforms lack mRNA IREs.[36]

IRON AND KIDNEY INJURY

Current understanding of systemic and cellular iron homeostasis is mainly derived from studies in hepatocytes, erythrocyte precursors, enterocytes and macrophages. Knowledge of physiological iron homeostasis in the kidney is limited,[25] despite observations made decades ago that iron could play a role in renal injury.[37-39] The kidney expresses many proteins that are not only essential for maintaining renal cellular iron balance,[40,41] but are also likely involved in maintaining systemic iron balance by renal reabsorption of filtered iron species.[25] However, the localization of several iron handling proteins in renal tubular epithelial cells in physiological conditions is still unclear. This includes their detailed intracellular localization, *i.e.* presence at the plasma membrane and/or in intracellular structures such as endosomes, as well as their localization in different tubular segments of the renal filter units, termed nephrons.[25] Moreover, human-based studies examining renal iron handling are scarce,[41] despite the fact that differences in systemic iron handling between animal models and human physiology have been recognized.[42] Although the kidney's primary function is to remove waste products from the circulation by urinary excretion,[43] evidence is growing that renal nephrons may function in iron homeostasis by preventing urinary iron and transferrin excretion regardless of systemic iron levels.[44] As such, renal iron handling may differentiate from iron handling in other cell types. Renal tubular cells express additional proteins that are shown to play a role in renal iron handling, *i.e.* the megalin:cubilin:amnionless receptor complex and NGAL receptor (NGALR, SLC22A17).[25] Moreover, iron handling differs between distinct segments of the renal nephron, e.g. between proximal and distal tubules (PTs and DTs, respectively).[25] Renal tubuli could be exposed to high iron levels in systemic iron overload disorders or kidney disease, which may have deleterious consequences in terms of iron toxicity. A better understanding of the mechanisms of human renal tubular iron handling and subsequent toxicity will be essential in dissecting the role of iron in kidney injury.

Renal iron homeostasis

Plasma molecules can enter the nephron by glomerular filtration into the renal tubular lumen. Subsequently, essential molecules are reabsorbed back into the circulation by reabsorption by renal tubules, whereas waste products are excreted in urine.[43] TBI in the systemic circulation is suggested to be at least partly filtered by the glomerulus. *In vivo* studies have demonstrated the presence of transferrin in the primary urine, and showed that ~0.4 mg of iron is daily filtered by the rat kidney, of which only 0.7% is daily excreted in the final urine.[40,45,46] Indeed, hardly any iron or transferrin is found in urine in human healthy volunteers,[14,47-49] suggesting that filtered TBI is almost completely reabsorbed by the renal tubular epithelium. Although proteins filtered by the glomerulus are predominantly reabsorbed by PTs,[25] micropuncture studies in rats demonstrated that both

PTs and DTs can reabsorb TBI.[45,46] The TBI uptake transporter TfR1 is found in the kidney, but its intracellular localization in renal tubules remains controversial. Studies in mice, rats and human kidney tissues showed TfR1 immunostaining in PTs and DTs at both the apical and basolateral tubular membranes.[44,50-54] TBI uptake in PTs can also be mediated by the endocytic megalin:cubilin:amnionless receptor complex.[55] Studies in megalin deficient mice and dogs with deficient cubilin surface expression showed that transferrin is a ligand for the cubilin receptor, but transferrin endocytosis requires cooperation of both megalin and cubilin.[56] Function of this receptor complex also requires the transmembrane protein amnionless, which is essential for cubilin trafficking towards the apical membrane.[57,58] In addition, TBI uptake by this receptor complex is supported by findings of increased urinary transferrin levels in patients with Fanconi syndrome, a disorder characterized by compromised PT reabsorption by the megalin:cubilin:amnionless receptor complex,[48,59] or Donnai-Barrow/Facio-Oculo-Acustico-Renal syndrome, an extremely rare disorder characterized by megalin deficiency.[60] Internalization of fluorescently labeled TBI was also shown in mouse distal tubular epithelial cells, facilitated by the NGALR.[61] Subsequent to endocytosis by any of the abovementioned endocytic transporters, iron is transported from the endosome towards the cytosolic, but this process has not yet been studied in renal tubular epithelial cells. Although DMT1 and ZIP14 protein expression have been detected in murine kidney (DMT1;[40,62-68] ZIP14[69]) and ZIP8 was found in mice and human kidney,[69-71] functional studies on their role in renal tubular TBI-derived iron uptake are absent. Similar to other cell types, intracellular iron is stored in ferritin and iron is exported by ferroportin. Although ferroportin was localized to the tubular basolateral membrane by several studies in mice and rats,[65,72-74] this has not been examined in the human kidney before. Interestingly, a study in mice detected ferroportin on the apical tubular membrane and suggested a role of this protein in iron import.[75]

DISORDERS OF SYSTEMIC IRON OVERLOAD

Renal injury as a result of chronic iron exposure is an emerging avenue of interest in iron overload disorders. Disturbed intestinal iron uptake is known to result in systemic iron overload, which may cause injury to organs including the liver and heart,[9] but has also been associated with renal injury.[41] Increased iron levels reside in the systemic circulation but are also deposited in parenchymal tissues.[9] In hereditary hemochromatosis (HH), unrestricted iron uptake that is inappropriate to body iron stores usually results from inherited defects in proteins involved in hepcidin regulation.[76] The most common form in Caucasians (>90%) is related to a homozygous variant in the human hemochromatosis gene (*HFE*, HFE-HH*)* on chromosome 6 (p.Cys282Tyr/p.Cys282Tyr, abbreviated C282Y/C282Y).[77] HFE-HH affects 1 in 200-300 individuals of Northern European descent,[78-80] but its clinical penetrance is low, only 13.5% in a meta-analysis of 19 epidemiological

studies,[78] indicating many individuals carry the genotype without experiencing iron overload symptoms. In practice, patients are mostly males between 40-60 years of age.[78] HFE-HH results in innate low hepcidin levels,[81] which can lead to iron loading in the liver, heart, pituitary and pancreas and subsequent liver cirrhosis, hepatocellular carcinoma and cardiac myopathy.[82,83] Treatment for patients with HFE-HH usually consists of phlebotomy (venesection), when large quantities of iron in erythrocytes are removed.[78,80] Subsequently, circulating iron is replenished by iron stored in tissues to provide iron for erythropoiesis, leading to a gradual decline in body iron stores.[81]

Systemic iron overload can also be secondary to other disorders (acquired), which is the case for inherited hemoglobin disorders, including thalassemia syndromes, sickle cell disease, sideroblastic anemia or other forms of anemia characterized by ineffective erythropoiesis.[84] In thalassemia disorders, reduced production of α- or β-globin chains, causing α- or β-thalassemia, respectively, results in disturbed hemoglobin synthesis and, as such, impaired erythropoiesis.[85] The epidemiology of thalassemia disorders is not completely defined, although a relevant high prevalence is found in the Mediterranean area, Middle East, sub-Sahara Africa, Indian subcontinent and Southeast Asia.[86,87] Patients with β-thalassemia are typically classified according to their level of globin production, anemia severity and clinical presentation.[84] Patients with β-thalassemia minor carry a heterozygous mutation, but are clinically asymptomatic. Whereas β-thalassemia intermedia can present with variable mutations and treatment requirements, patients suffering from β-thalassemia major usually present with severe anemia in infancy and become transfusion dependent for life.[84] In β-thalassemia major, ineffective erythropoiesis cause anemia and, as such, lower hepatic hepcidin production triggers increased intestinal iron uptake and increased iron release from reticuloendothelial macrophages.[88] Moreover, additional frequent red blood cell transfusions as treatment of anemia further add to systemic iron levels.[84] Iron loading in the heart, liver and endocrine organs causes complications of cardiomyopathy, chronic hepatitis and endocrine problems.[89] Iron removal therapy in patients with β-thalassemia consists of treatment with iron chelation medication.[84] Several iron chelators are available that bind iron in the circulation and subsequently cause urinary and/or fecal excretion of the iron-chelator complexes, reducing systemic iron levels.[90] When chelation therapy is initiated at an early age, this treatment is successful in reducing iron-related complications.[91]

In systemic iron overload, the amount of iron that is bound to circulating transferrin (transferrin saturation, expressed as percentage) is used as measure for circulating iron overload.[76] Whereas transferrin is saturated for 20-45% with iron in health, this value usually exceeds 60% in patients with systemic iron overload.[80] Plasma ferritin levels correlate with body iron stores, and are, therefore, used as diagnostic tool to determine iron stored in parenchymal tissues and reticuloendothelial macrophages.[80,84] When transferrin is for at least 70% saturated with iron, also non-transferrin-bound iron

Table 1.1: Iron overload phenotype and treatment in *HFE*-related hereditary hemochromatosis and β-thalassemia major summarized

	***HFE*-related hereditary hemochromatosis**	**β-thalassemia major**
Cause of iron loading	Unrestricted intestinal iron uptake due to innate low hepcidin levels	Unrestricted intestinal iron uptake due to ineffective erythropoiesis + red blood cell transfusions
Main localization of iron deposition	Liver, heart, endocrine organs	Heart, liver, endocrine organs
Age of onset	40 – 60 years	Infancy
Predominant treatment to reduce systemic iron levels	Phlebotomy	Iron chelation

(NTBI) where iron is bound to low-molecular weight carrier molecules, can be detected.[92,93]Although the exact nature of NTBI remains to be determined and is suggested to vary based on the degree and duration of iron overload,[92-94] iron in NTBI is predominantly bound to citrate, but can also bind to acetate or albumin.[95-97] Iron binds its ligands in NTBI with a much lower affinity than in TBI, rendering it potentially reactive.[92] Specific laboratory analyses are designed to determine the labile and redox active fraction of NTBI, termed labile plasma iron (LPI).[8,98]

The iron phenotype and treatment options of patients with HFE-HH or β-thalassemia major are summarized in Table 1.1.

Renal dysfunction in systemic iron overload

Historically, survival of patients with β-thalassemia major was limited as a result of severe cardiomyopathy, which is attributed to the limited ability of cardiomyocytes to cope with internalized LPI and subsequent iron accumulation and ROS production.[99] Since the discovery of iron chelation therapy 50 years ago, morbidity and mortality rates in β-thalassemia have seriously declined.[91,100,101] However, extending patient survival with the current treatment possibilities has resulted in reports of renal dysfunction in patients with thalassemia syndromes.[102] This includes glomerular anomalies of increased urinary creatinine clearance and proteinuria[103] but also tubular dysfunction, manifested by hypercalciuria, hyperuricosuria and renal glycosuria.[104-106] More specifically, tubular dysfunction was localized to PTs, by reports of increased urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) and β-2-microglobulin, indicators of PT dysfunction.[104,105,107-110] Multiple clinical and preclinical studies show that these renal injury findings could be associated with chronic exposure to increased renal iron concentrations. Indeed, in patients with β-thalassemia syndromes, histological staining of MRI imaging showed iron accumulation in the kidney.[109,111,112] Moreover, increased urinary iron levels were also observed,[113-116] indicating the nephrons are exposed to high iron levels. In heterozygous β-globin knockout mice, a mouse model for β-thalassemia intermedia, severe systemic iron

loading induced renal iron accumulation.[117] In other systemic iron overload mouse models, iron was also found to deposit in the kidney. In HFE or HFE2 knockout mice, mouse models for HH, increased iron levels were detected in the kidney[24] Moreover, when systemic iron overload was induced in control mice by long term high iron diet, iron dextran injection or administration of radioactive iron (^{59}TBI and ^{59}Fe-NTBI), iron accumulated in the kidneys, as shown by Perls' staining, iron quantification or radioactive signal detection.[118-125] Reports in patients with HFE-HH seem to confirm the above mentioned *in vivo* findings, since renal iron accumulation and enhanced urinary iron levels were reported in these patients.[126-133] All together, this evidence supports an association between chronic exposure to increased renal iron levels and renal injury in systemic iron overload.

Currently, it remains unknown which molecular mechanisms are involved in human renal iron loading during systemic iron overload and potential subsequent iron-induced injury. Similar to observations in cardiomyocytes and hepatocytes,[134,135] chronic exposure to high iron concentrations is shown to induce oxidative stress in renal tubular epithelial cells. Moreover, in human and porcine tubular epithelial cells, 72h iron exposure was found to reduce cell viability and decrease cell proliferation, respectively.[136,137] Studies in rats have shown that a long term iron diet can result in tubular atrophy, iron deposition and enhanced malondialdehyde (MDA) formation, a byproduct of lipid peroxidation induced by oxidative stress, in the renal tubular epithelium.[122,138] Moreover, iron-induced lipid peroxidation and kidney dysfunction were ameliorated in iron-overloaded rats by administration of antioxidant compounds,[139-141] suggesting that oxidative stress may cause iron overload-induced renal injury. Patients with HFE-HH or β-thalassemia major also showed increased MDA levels and decreased levels of antioxidants vitamin E and ascorbic acid in plasma, as well as increased oxidized RNA products in urine.[107,142-146] These findings suggest that chronically increased renal tubular iron exposure can induce oxidative stress-related renal tubular injury. Mechanistically, uptake of TBI (as described above) and NTBI can contribute to renal iron loading and subsequent renal injury. Divalent metal transporters ZIP8, ZIP14 and DMT1 are implicated in NTBI transport at the plasma membrane in hepatocytes, pancreatic cells and neurons (Figure 1.3),[21,23,147-151] but the role of these transporters in NTBI uptake has not been studied in renal tubular epithelial cells.

CHRONIC KIDNEY DISEASE

In chronic kidney disease (CKD), iron is suggested to play a detrimental role in the onset or progression of renal injury.[152] CKD is a heterogeneous group of kidney disorders characterized by a decrease in glomerular filtration rate (GFR) <60 ml/min/1.73m^2 in combination with pathologic kidney abnormalities on biopsy, imaging or urinary sediment or proteinuria for 3 months or more.[153] In these patients, valuable serum proteins are lost

in urine as a result of glomerular leakage whereas waste products from the human body are not properly removed from the circulation by the renal tubular epithelial cells.[43] Currently, CKD affects 13% of the population worldwide.[154] As kidney function gradually decreases over time, these patients can progress to end stage renal disease, which has a yearly mortality rate above 20%.[155] Current treatment for patients with CKD is mainly aimed at ameliorating the symptoms of nephropathy, including proteinuria, which is the major risk factor for CKD progression.[156] Treatment to reduce proteinuria consists of medication for blood pressure control, for instance by use of angiotensin-converting enzyme (ACE) inhibitors.[157] Unfortunately, this treatment is not effective in reducing proteinuria in a large proportion of CKD patients, and progression to end stage renal disease or the emergence of cardiovascular complications cannot be prevented.[158] Therefore, new treatment modalities are required for patients with CKD, preferably aimed at the underlying pathological mechanisms.

Iron-mediated injury in chronic kidney disease

In many pathologies of CKD, renal disease is initiated by injury to the glomerulus, which manifests as proteinuria.[159] As a result, increased levels of circulating TBI are filtered into the tubular lumen and result in high iron exposure for renal tubular epithelial cells. Indeed, increased urinary transferrin levels have been detected in patients with various CKD pathologies.[38,48,49,160] However, in renal tubulointerstitial disorders, also called tubulopathy, renal disease originates from abnormalities in the renal tubuli. These disorders may affect TBI handling, *i.e.* TBI uptake, based on the increased urinary transferrin excretion found in patients with Fanconi syndrome, a PT tubulopathy disorder.[48] Injury to both the glomerulus and tubulointerstitium are recognized as part of the progression of CKD.[161] As such, both glomerular TBI filtration and TBI reabsorption can be disturbed in disorders of CKD.

Accumulating evidence suggests that renal iron accumulation may be involved in the onset and/or progression of CKD. The first reports of a potential role of iron in the pathogenesis of CKD date from the 1980s, when iron reduction by a low iron diet or by infusion of an iron chelator was shown to prevent renal function deterioration in nephrotoxic serum nephritis.[37] This evidence was extended with the finding that iron reduction was also able to ameliorate renal tubular injury in an animal model of membranous nephropathy.[162] In the years that followed, increased renal iron accumulation and urinary iron excretion were also reported in various other CKD animal models, such as puromycin aminonucleoside- and adriamycin-induced nephrotic syndrome, 5/6 nephrectomy, hypertension nephropathy, diabetic nephropathy, and passive Heymann nephritis.[163-169] More recently, renal iron accumulation was also found in an animal model of lupus nephritis.[170] Altogether, this illustrates that renal tubular epithelial cells are likely to be exposed to high iron concentrations and develop

tubulointerstitial injury in a large variety of CKD pathologies. Interestingly, administration of an iron chelator or low-iron diet was reported to reduce proteinuria and halt the loss of kidney function in multiple animal models of CKD.[162,165-168,170,171] These findings suggests a relation between renal iron deposition, urinary iron excretion and tubular injury in several CKD animal models. Increased urinary iron excretion has also been observed in patients with CKD.[38,49,160,172-178] Moreover, iron deposition was found in biopsies of various nephrotic CKD disorders, including IgAN nephropathy, membranous nephropathy and diabetic nephropathy.[39,179,180] More specifically, iron deposition was localized to PTs lysosomes in nephrotic CKD, which was present in higher levels in damaged renal tubuli than in tubules with less damage.[181] Although this suggests that increased iron exposure may be deleterious for renal tubuli in patients with nephrotic CKD, insights in the molecular mechanisms of tubular iron handling in response to enhanced iron supply in human CKD are lacking.

In addition to the uncertainties of renal iron handling, our insights in the mechanisms of iron toxicity are far from complete. Increased oxidative stress production and decreased mRNA expression of antioxidant enzymes were reported in mouse models of diabetic nephropathy and protein-overload induced tubulointerstitial injury,[166,182,183] as well as production of inflammatory cytokines, ultimately leading to renal fibrosis.[168,184] An iron-restricted diet ameliorated oxidative stress formation and inflammation in several studies,[166,168,182] indicating that both oxidative stress and inflammation are key processes involved in iron-induced renal injury during CKD. However, the molecular mechanisms involved in these detrimental processes, as well as potential interaction between these pathways during iron-mediated tubular injury remain elusive.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis was to gain insights into the molecular mechanisms of renal tubular iron handling in health and disease and to improve our understanding of the detrimental role of iron in renal injury.

To this end, we performed studies to assess tubular iron handling in human health, systemic iron overload and CKD. In **Chapter 2**, we describe human renal iron handling for which we measured urinary iron excretion and tubular injury in healthy controls, patients with tubular dysfunction and patients with systemic iron overload. In **Chapter 3**, we characterized the molecular mechanisms involved in TBI and NTBI uptake in conditionally immortalized proximal tubular epithelial cells (ciPTECs), focusing on divalent metal transporters ZIP8 and ZIP14. In **Chapter 4**, we describe our studies into the potential of chronic iron overload to induce cytotoxicity in ciPTECs and investigated the molecular mechanisms involved. Lastly, in **Chapter 5**, we examined the extent and localization of iron deposition in renal biopsies of patients with various CKD pathologies. Here, we also characterized the presence and localization of proteins involved in iron handling in healthy controls and CKD patients, as well as injury related to iron accumulation. The implications and future prospects of the findings described in this thesis are discussed in **Chapter 6** and summarized in **Chapter 7**.

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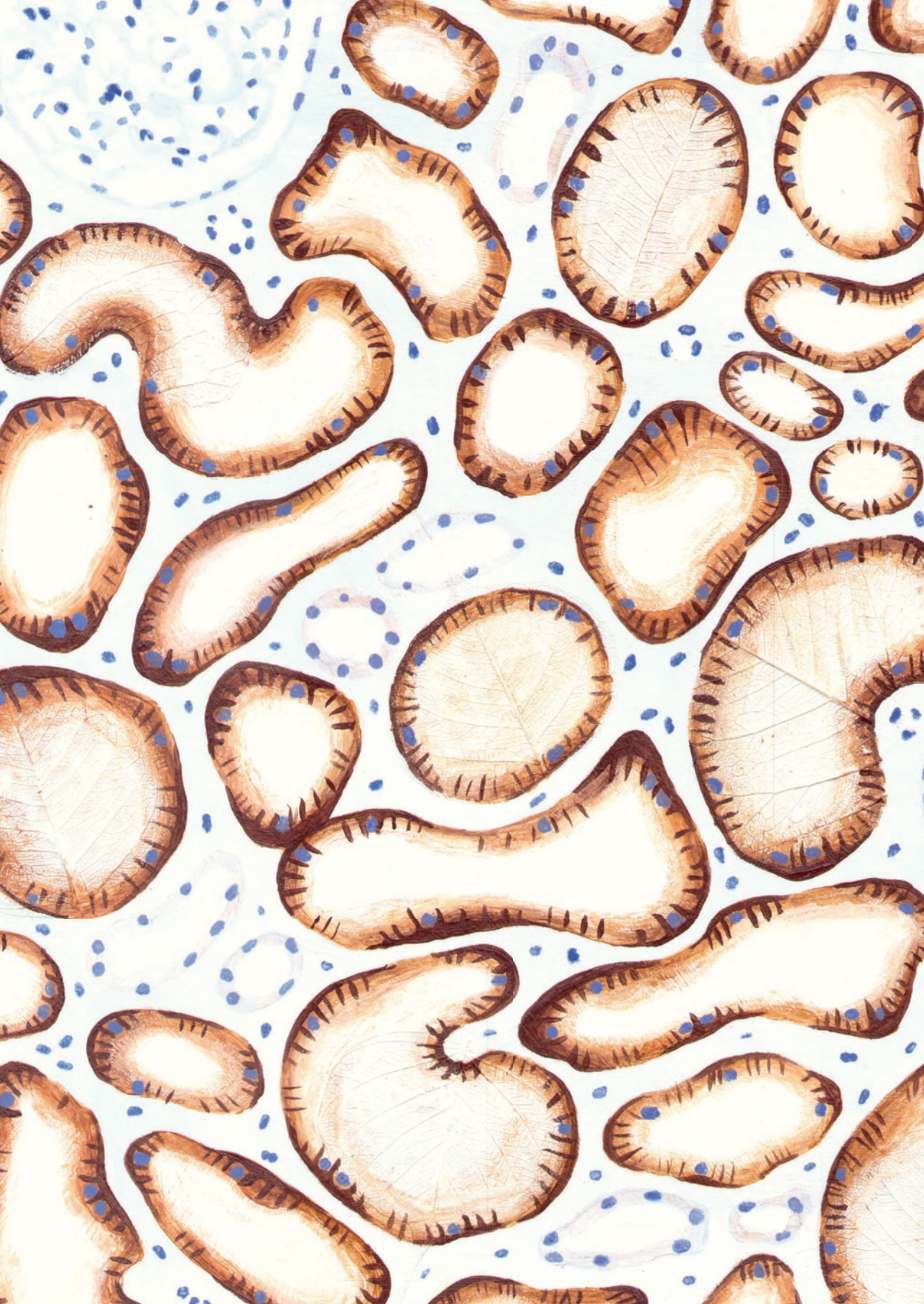

2

Iron handling by the human kidney: Glomerular filtration and tubular reabsorption both contribute to urinary iron excretion

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ABSTRACT

Background: In physiological conditions, circulating iron can be filtered by the glomerulus and is almost completely reabsorbed by the tubular epithelium to prevent urinary iron wasting. Increased urinary iron concentrations have been associated with renal injury. However, it is not clear whether increased urinary iron concentrations in patients are the result of increased glomerular iron filtration and/or insufficient tubular iron reabsorption and if these processes contribute to renal injury.

Methods: We measured plasma and urine iron parameters and urinary tubular injury markers in healthy human subjects (n=20), patients with systemic iron overload (n=20) and patients with renal tubular dysfunction (n=18).

Results: Urinary iron excretion parameters were increased in both patients with systemic iron overload and tubular dysfunction, whereas plasma iron parameters were only increased in patients with systemic iron overload. In patients with systemic iron overload, increased urinary iron levels associated with elevated circulating iron, as indicated by transferrin saturation (TSAT), and increased body iron, as suggested by plasma ferritin concentrations. In patients with tubular dysfunction, enhanced urinary iron and transferrin excretion were associated with distal tubular injury as indicated by increased urinary glutathione s-transferase-pi-1-1 (GSTP-1-1) excretion. In systemic iron overload, elevated urinary iron and transferrin levels were associated with increased injury to proximal tubules, indicated by increased urinary kidney injury marker 1 (KIM-1) excretion.

Conclusion: Our explorative study demonstrates that both glomerular filtration of elevated plasma iron levels and insufficient tubular iron reabsorption could increase urinary iron excretion and cause renal injury.

INTRODUCTION

In physiological conditions, circulating iron is bound to transferrin (transferrin-bound iron, TBI), which can be filtered by the glomerulus of the kidney into the renal tubular lumen.[1] Subsequently, filtered TBI is thought to be almost completely reabsorbed by renal tubular epithelial cells, since hardly any transferrin or iron is found in urine of healthy volunteers.[1-3] In addition, micropuncture studies in rats demonstrated the presence of transferrin in primary urine and showed iron can be reabsorbed by both the renal proximal tubule (PT) and distal tubule (DT).[4]

Increased iron exposure can be harmful for tubular epithelial cells, because iron is known to catalyze reactive oxygen species formation in the Fenton reaction and cause tissue injury.[5] In human and porcine PT epithelial cells, iron exposure decreased cellular viability and proliferation and caused oxidative cellular injury.[6-8] Moreover, increased urinary iron levels have been found in patients with nephrotic syndrome and diabetic nephropathy,[3,9-13] suggesting an association between enhanced urinary iron concentrations and renal injury. In animal models of renal diseases, such as minimal change nephrotic syndrome and nephrotoxic serum nephritis, increased urinary iron excretion coincided with renal tubular injury.[14-16] Currently, it is not clear whether increased urinary iron concentrations are the result of increased glomerular iron filtration and/or insufficient tubular iron reabsorption and if these processes directly contribute to the renal tubular injury.

During systemic iron overload, unrestricted iron intake from the intestine in HFE-hereditary hemochromatosis (HFE-HH) or additional frequent red blood cell transfusions in β-thalassemia major increase circulating TBI and also result in the presence of non-transferrin-bound iron (NTBI), when the iron binding capacity of transferrin is exceeded.[17,18] Moreover, these patients also present with high plasma ferritin concentrations, reflecting iron deposition in parenchymal tissues, like the liver and heart.[17] In β-thalassemia major, elevated plasma ferritin levels were also associated with renal iron deposition.[19] Increased urinary iron concentrations have been observed in patients with systemic iron overload,[20-22] suggesting that glomerular filtration of increased circulating iron exceeds the tubular iron reabsorption capacity in these patients. Alternatively, urinary iron excretion can result from insufficient iron reabsorption in either PTs or DTs. Filtered proteins, including TBI,[23,24] are predominantly reabsorbed by PTs,[25] but also DTs express transporters involved in iron handling and are reported to take up TBI.[26,27] Increased urinary transferrin levels in patients with Fanconi syndrome, characterized by compromised proximal tubular reabsorption,[1] suggest the tubular reabsorption capacity affects urinary transferrin levels, and similarly, possibly also urinary iron levels.

To better understand the contribution of increased glomerular iron filtration and/or insufficient tubular iron reabsorption by PTs and DTs to urinary iron levels and related renal injury, we performed an explorative study and measured plasma iron

parameters, urinary iron parameters and renal tubular injury markers among healthy subjects, patients with systemic iron overload and patients with renal tubular dysfunction.

MATERIALS AND METHODS

Study design

This explorative, observational study included subjects between November 2016 and October 2017. Healthy volunteers (n=20) were recruited at the Radboud university medical center (Radboudumc; Nijmegen, the Netherlands). Exclusion criteria included presence of renal disease, iron overload disorder or urinary tract infection, use of chelation medication or active menstruation. Patients with systemic iron overload disorders (n=20; Table 2.1) were included based on the diagnosis made by their treating physician and with most recent determined transferrin saturation (TSAT)>70% at Radboudumc and Amsterdam university medical center (Amsterdam, the Netherlands). Patients with renal tubular dysfunction disorders (n=18; Table 2.1) were included based on the diagnosis made by their treating physician in Radboudumc, Erasmus Medical Center (Rotterdam, the Netherlands), and UCL Centre for Nephrology, Royal Free Hospital (London, United Kingdom). This study was approved by the local ethics committee and performed according to national legislation and the declaration of Helsinki. All subjects signed informed consent forms.

Patients with HFE-HH were undergoing maintenance phlebotomies. Patients with β-thalassemia major on iron chelation medication withdrew the use of this medication for at least 4 days, to prevent bias in urinary iron. Plasma levels of deferasirox (DFX), used by six patients with β-thalassemia major, were determined as described in De Francia *et al.*[28] Urinary iron levels were corrected for minimal residual iron bound to DFX in three patients. Since deferoxamine and deferiprone (used by two and one β-thalassemia major patients, respectively) have a shorter half life than DFX, we anticipate that suspending these chelators for four days eliminated plasma concentrations and subsequent urinary excretion of potential iron-chelator complexes.

Sample collection

Heparin plasma and urine were collected, aliquoted and stored at -80°C. For analysis of glutathione s-transferase-pi-1-1 (GSTP1-1), urine samples were mixed with a 10% (v/v) storage buffer (1M HEPES pH 7.5, 5% bovine serum albumin, 1% sodium azide, 1% Tween-20, 10% glycerol) within 30 minutes of collection.

Laboratory analyses

Iron, total iron binding capacity (TIBC; calculated as transferrin (g/l)*25.0), creatinine, C-reactive protein (CRP), ferritin, creatinine, transferrin, and albumin analyses were performed according to routine diagnostic protocols at the Radboud Laboratory for Diagnostics at Radboudumc. Measurements of NTBI and labile plasma iron (LPI), the pathologically most relevant fraction of NTBI,[5] were performed according Zhang *et al* (1995)[29] and Esposito *et al* (2003),[30] respectively. TSAT (the percentage of circulating transferrin that is saturated with iron (calculated as iron/TIBC*100), NTBI and LPI were used as indicators of circulating iron species.[17,18] Estimated glomerular filtration rate (eGFR) was determined using the modifications of diet in renal disease (MDRD) formula. Urinary iron analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) at Maastricht University Medical Center (the Netherlands). For analysis of urinary oxidative stress, thiobarbituric acid (TBA) reactive substances (TBARs) were measured based on a previously described method.[31] In short, samples were mixed with 75 mM TBA in 75 mM KH_2PO_4 and heated to 96°C for 1h. After cooling, samples were mixed with butanol and centrifuged at low speed. Fluorescence of the supernatant was compared to a standard curve made of tetramethoxypropane. Kidney injury marker 1 (KIM-1; R&D Systems, DKM100) and GSTP1-1[32] were measured by ELISA. Plasma and urinary hepcidin concentrations were determined by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry as previously described.[33] All urine parameters were corrected for urinary creatinine concentration.

Table 2.1: Subject characteristics

	Healthy controls	Renal tubular dysfunction	Systemic iron overload
Subjects (n)	20	18	20
Age (years)	37.4 ± 3.4	41.9 ± 4.2	50.1 ± 4.2*
Gender (M/F)	12/8	17/1	14/6
Type of disorder	-	Cystinosis, n=2 Dent's disease, n=3 Fanconi syndrome, n=3 Nephronophthisis, n=1 Tubulo-interstitial nephritis, n=1 Wilson's disease, n=1 Secondary to chemotherapy, n=5 Lithium-induced nephrogenic diabetes insipidus, n=1 Unknown cause, n=1	β-thalassemia major, n=9 Diamond Blackfan anemia, n=1 HFE-related hereditary hemochromatosis, n=10

Age presented as mean ± standard error of the mean (SEM). *, p<0.05, compared to healthy controls by Mann Whitney U test.

F, female; M, male.

Statistical analysis

Data were statistically analyzed using SPSS 22 (IBM) and presented as median and interquartile range (IQR). One patient with tubular dysfunction was excluded from urinary analysis due to low creatinine concentration and plasma analysis could not be performed in four patients with tubular dysfunction as plasma samples were absent. Data were analyzed by Mann Whitney U test and non-parametric Spearman correlation coefficients. A p-value <0.05 was considered statistically significant.

RESULTS

Clinical and laboratory characteristics

Clinical characteristics of all study participants are listed in Table 2.1 and results of laboratory analyses in Table 2.2. Plasma CRP was within reference range (<10 mg/ml) for all groups, indicating plasma iron parameters were not biased by the presence of inflammation. Subjects with systemic iron overload were older than healthy controls (50.1 ± 4.2 years vs. 37.4 ± 3.4 years, $p<0.05$), but this was not the case for patients with renal tubular dysfunction (41.9 ± 4.2 years, ns). All three groups comprised predominantly male subjects (Table 2.1).

Increased urinary excretion of iron, transferrin and NTBI in patients with systemic iron overload

Plasma iron, TSAT, NTBI, LPI and ferritin were increased in patients with systemic iron overload compared to healthy controls, whereas TIBC, hepcidin and thehepcidin/ferritin ratio were decreased (ferritin $p<0.05$, hepcidin and hepcidin/ferritin ratio $p<0.01$, others $p<0.001$; Table 2.2), confirming low hepcidin-induced iron overload. Urinary iron and NTBI levels were significantly increased in patients with systemic iron overload compared to healthy controls ($p<0.01$ and $p<0.05$, respectively; Table 2.2). Urinary iron levels correlated moderately with plasma TSAT ($r=0.47$, $p<0.05$; Figure 2.1a), but not with plasma iron or TIBC alone (Figure 2.1b, c). These findings indicate that urinary iron excretion could be caused by glomerular filtration of increased circulating iron levels. Moreover, both urinary iron and NTBI concentrations correlated strongly with plasma ferritin levels ($r=0.75$, $p<0.001$, and $r=0.71$, $p<0.001$; Figure 3.1d, e), suggesting that the increased urinary iron excretion could also be associated with high iron levels deposited in renal tubuli.

Table 2.2: Results of laboratory analyses in the 3 study groups

		Healthy controls	Renal tubular dysfunction	Systemic iron overload
Plasma	Iron (µmol/l)	16.0 (12.7 – 18.7)	15.0 (12.5 – 21.5)	30.5 (27.5 – 39.5)***
	TIBC (µmol/l)	64.0 (57.2 – 73.0)	60.0 (53.7 – 74.7)	44.5 (41.2 – 48.0)***
	TSAT (%)	24.5 (20.1 – 30.8)	24.4 (17.2 – 33.6)	74.9 (62.5 – 89.7)***
	Ferritin (µg/l)	121.0 (68.0 – 178.5)	179.5 (38.5 – 269.8)	371.0 (111.0 – 906.8)*
	NTBI (µmol/l)	0.23 (0.23 – 0.23)	0.23 (0.23 – 0.23)	1.22 (0.72 – 2.32)***
	LPI (µmol/l)	0.11 (0.10 – 0.14)	0.15 (0.11 – 0.22)	0.24 (0.22 – 0.31)***
	CRP (mg/ml)	0.5 (0.5 – 0.5)	1.5 (0.5 -2.2)	1.0 (0.5 – 2.0)
	Hepcidin (nM)	5.1 (1.3 – 7.1)	7.3 (1.1 – 10.3)	0.8 (0.3 – 2.0)**
	Hepcidin/ferritin ratio (nmol/µg)	36.9 (20.7 – 50.4)	37.9 (15.4 – 43.6)	2.3 (1.4 – 6.1)**
Urine	Iron (µmol/g creatinine)	0.03 (0.02 – 0.07)	0.09 (0.03 – 0.20)*	0.21 (0.05 – 2.39)**
	Transferrin (mg/g creatinine)	1.0 (0.6 – 2.7)	3.0 (0.9 – 11.8)*	1.1 (0.6 – 10.8)
	NTBI (µmol/g creatinine)	0.4 (0.2 – 0.7)	0.6 (0.4 – 1.6)	0.5 (0.2 – 1.7)*
	Hepcidin (nM/g creatinine)	20.5 (6.9 – 49.7)	16.5 (8.7 – 56.0)	2.9 (1.3 – 11.1)**
	Albumin (mg/g creatinine)	3.8 (2.8 – 5.7)	27.5 (9.2 – 168.5)***	13.1 (7.1 – 283.8)***
	KIM-1 (ng/mmol creatinine)	0.3 (0.1 – 0.5)	0.7 (0.4 – 0.8)**	0.9 (0.6 – 2.2)***
	GSTP1-1 (ng/mmol creatinine)	3.1 (1.8 – 6.2)	7.6 (2.1 – 65.6)*	2.6 (0.9 – 9.3)
	TBARs (µM/g creatinine)	1.4 (1.1 – 1.7)	1.4 (1.0 – 1.8)	2.5 (1.8 – 3.0)***
eGFR (ml/min/1.73m^2)		90.0 (67.8 – 100.8)	52.4 (34.6 – 80.1)***	76.8 (64.0 – 109.5)

Results presented as median (interquartile range (IQR)). *, p<0.05; **, p<0.01; ***, p<0.001 compared to healthy controls by Mann Whitney U test. eGFR calculated by MDRD formula. One healthy control and one patient with systemic iron overload were excluded from NTBI analysis and one patient with tubular dysfunction was excluded from urinary KIM-1 analysis.

CRP, C-reactive protein; eGFR, estimated glomerular filtration rate; GSTP1-1, glutathione s-transferase-pi-1-1; KIM-1, kidney injury marker 1; LPI, labile plasma iron; NTBI, non-transferrin-bound iron; TBARs, thiobarbituric acid (TBA) reactive substances; TIBC, total iron binding capacity; TSAT, transferrin saturation.

We subsequently stratified patients with HFE-HH and β-thalassemia major to examine urinary iron excretion specifically in these two distinct iron overload disorders

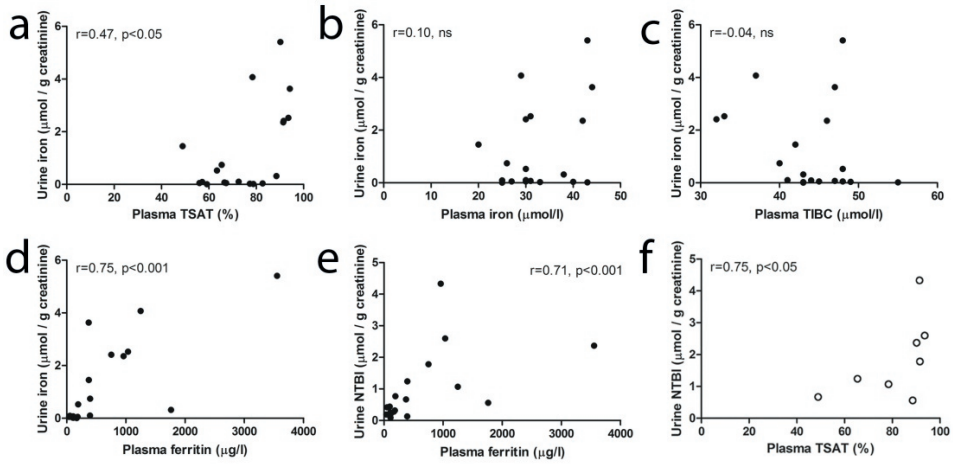

Figure 2.1: Urinary iron and non-transferrin-bound iron (NTBI) correlate with plasma iron parameters in systemic iron overload

Correlation between urinary iron excretion and plasma transferrin saturation (TSAT) (a), iron (b), total iron binding capacity (TIBC) (c) and ferritin (all n=20) (d); urinary NTBI and plasma ferritin in patients with systemic iron overload (n=19) (e; all filled circles); and urinary NTBI and plasma TSAT in patients with β-thalassemia major (n=8; urinary NTBI value missing for one patient) (f; open circles). Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value or not significant (ns)).

(Table 3.3). Patients with HFE-HH, but not with β-thalassemia major, were significantly older than healthy controls ($p<0.001$) and predominantly consisted of males, whereas both genders were equally present in β-thalassemia major. Plasma iron levels were increased in both HFE-HH and β-thalassemia major compared to healthy controls (both $p<0.001$) and both patient groups showed low hepcidin/ferritin ratios in line with their HFE-genotype and ineffective erythropoiesis, respectively.[34,35] However, β-thalassemia major patients showed a more severe increase in plasma TSAT and NTBI and decrease in TIBC (all $p<0.05$ compared to HFE-HH). Moreover, ferritin was increased in β-thalassemia major only ($p<0.001$ compared to both control and HFE-HH). These findings suggest that both circulating and tissue iron overload are more pronounced in patients with β-thalassemia major than in HFE-HH patients in our study cohort, confirming previous findings.[18] We found that urinary iron, transferrin and NTBI concentrations were only elevated in β-thalassemia major ($p<0.001$, $p<0.05$ and $p<0.01$, respectively), whereas urinary NTBI strongly correlated with plasma TSAT in these patients ($r=0.75$, $p<0.05$, Figure 2.1f). This demonstrates that only severely elevated iron parameters, as seen in β-thalassemia major, are associated with elevated urinary iron parameters.

Table 2.3: Laboratory results in subjects with HFE-related hereditary hemochromatosis and β-thalassemia major

		HFE-related hereditary hemochromatosis	β-thalassemia major
Subjects (n)		10	9
Age (y)		63.8 ± 3.7***	37.4 ± 4.5
Gender (M/F)		9/1	5/4
Plasma	Iron (µmol/l)	30.0 (26.5 – 34.7)***	31.0 (27.5 – 42.5)***
	TIBC (µmol/l)	46.0 (43.0 – 48.2)***	42.0 (35.0 – 46.5)***, #
	TSAT (%)	66.9 (58.7 – 77.6)***	90.2 (71.9 – 92.6)***, #
	Ferritin (µg/l)	113.0 (85.0 – 185.3)	958.0 (383.0 – 1504.0)***, ###
	NTBI (µmol/l)	0.94 (0.43 – 1.25)***	2.31 (1.20 – 2.54)***, #
	LPI (µmol/l)	0.23 (0.19 – 0.24)***	0.31 (0.24 – 0.46)***, #
	Hepcidin (nM)	0.3 (0.3 – 0.7)***	1.7 (0.8 – 2.4)##
	Hepcidin/ferritin ratio (nmol/µg)	2.3 (1.5 – 8.8)***	2.3 (0.7 – 4.2)***
Urine	Iron (µmol/g creatinine)	0.1 (0.02 – 0.09)	2.4 (1.0 – 3.8)***, ###
	Transferrin (mg/g creatinine)	0.8 (0.6 – 1.3)	2.8 (1.1 – 26.4)*, #
	NTBI (µmol/g creatinine)	0.2 (0.1 – 0.4)	1.5 (0.7 – 2.5)**, ###
	Hepcidin (nM/g creatinine)	1.5 (0.7 – 2.8)***	8.1 (3.1 – 17.3)##
	Albumin (mg/g creatinine)	8.4 (5.2 – 13.5)**	23.9 (11.6 – 705.6)***, #
	KIM-1 (ng/mmol creatinine)	0.9 (0.5 – 1.0)**	2.2 (0.9 – 3.4)***, #
	GSTP1-1 (ng/mmol creatinine)	1.6 (0.7 – 8.8)	5.9 (1.7 – 10.1)
	TBARs (µM/g creatinine)	2.3 (1.8 – 2.6)***	3.1 (1.9 – 4.2)***
eGFR (ml/min/1.73m^2)		76.8 (62.8 – 99.3)	76.3 (62.1 – 129.0)

Results as median (interquartile range (IQR)). *, p<0.05; **, p<0.01; ***, p<0.001 compared to healthy controls (Table 3.2); #, p<0.05; ##, p<0.01; ###, p<0.001 compared to hereditary hemochromatosis by Mann Whitney U test. eGFR calculated by MDRD formula. One patient with β-thalassemia major was excluded from NTBI analysis.
eGFR, estimated glomerular filtration rate; GSTP1-1, glutathione s-transferase-pi-1-1; KIM-1, kidney injury marker 1; LPI, labile plasma iron; NTBI, non-transferrin-bound iron; TBARs, thiobarbituric acid (TBA) reactive substances; TIBC, total iron binding capacity; TSAT, transferrin saturation.

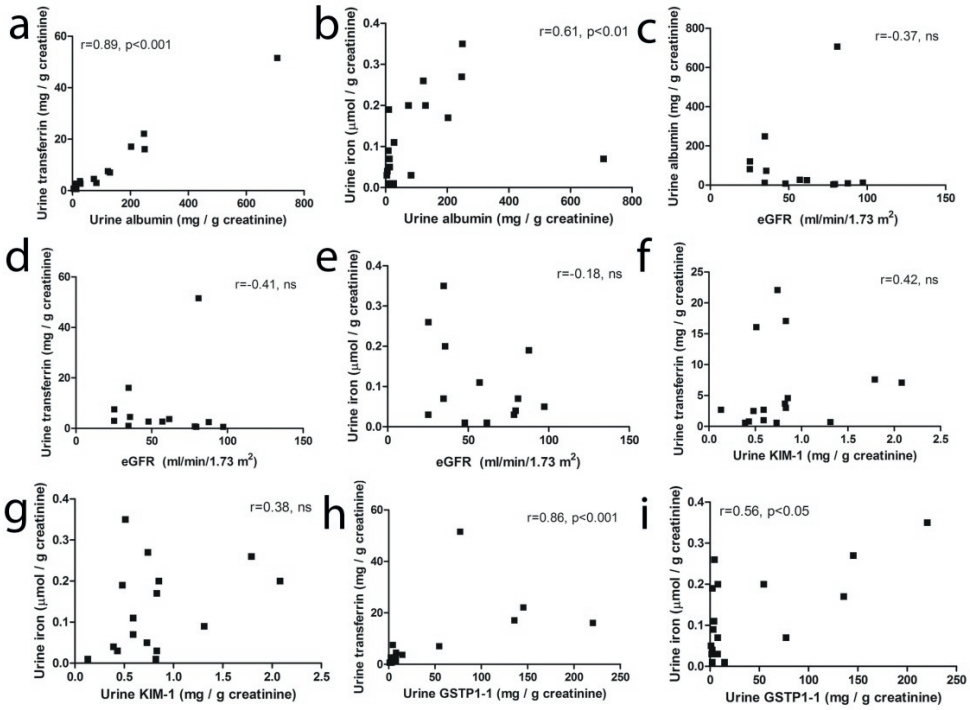

Figure 2.2: Urinary iron and transferrin correlate with urinary kidney parameters in tubular dysfunction
Correlation between urinary transferrin and iron with urinary albumin (n=17) (a, b); urinary albumin, transferrin and iron with estimated glomerular filtration rate (eGFR) (n=13) (c, d, e); urinary transferrin and iron with proximal tubular injury marker kidney injury marker 1 (KIM-1) (n=16; urinary KIM-1 value missing for one patient) (f, g) and distal tubular injury marker glutathione s-transferase-pi-1-1 (GSTP1-1) (n=17) (h, i) in patients with tubular dysfunction. Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value or not significant (ns)).

Increased urinary iron and transferrin excretion in patients with tubular dysfunction

Tubular dysfunction was shown by increased levels of PT injury marker KIM-1 and DT injury marker GSTP1-1 ($p<0.01$ and $p<0.05$ respectively, compared to healthy controls; Table 2.2). In these patients, urinary iron and transferrin, but not NTBI, were increased (both $p<0.05$) despite the fact that circulating iron homeostasis parameters were within reference range (iron, TSAT, NTBI, LPI, ferritin and hepcidin), suggesting that urinary iron loss did not result in decreased circulating and stored iron levels. These patients showed increased urinary albumin levels ($p<0.05$). Moreover, urinary transferrin and albumin levels strongly correlated ($r=0.89$, $p<0.001$; Figure 2.2a), thus indicating similar glomerular filtration and tubular handling of both proteins, as demonstrated before.[9] Interestingly, urinary iron levels also related to albuminuria ($r=0.61$, $p<0.01$; Figure 2.2b), whereas

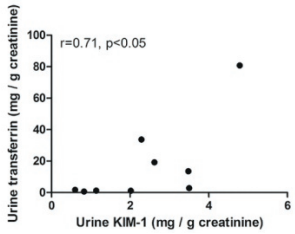

Figure 2.3: Urinary transferrin correlates with urinary proximal tubular injury in β-thalassemia major
Correlation between urinary transferrin and proximal tubular injury marker kidney injury marker 1 (KIM-1) in patients with β-thalassemia major (n=9). Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value).

albumin, transferrin nor iron levels in urine correlated with eGFR in these patients (Figure 2.2c, d, e), suggesting that insufficient tubular protein reabsorption, rather than increased glomerular filtration,[36,37] may cause elevated iron and transferrin excretion.

Moreover, we examined the relation between iron excretion and renal injury. Therefore, we assessed urinary TBAR concentrations, as a measure for urinary oxidative stress. We found that urinary TBARs were not increased compared to healthy controls (Table 2.2), suggesting that iron excretion did not readily result in oxidative stress in these patients. Subsequently, we studied the correlation between urinary iron and transferrin concentrations and KIM-1 and GSTP1-1 to investigate the relation between iron excretion and PT and DT injury, respectively.[38,39] Whereas we found no correlation between KIM-1 and urinary transferrin or iron levels (Figure 2.2f, g), GSTP1-1 was positively associated with both transferrin and iron ($r=0.86$, $p<0.001$, and $r=0.56$, $p<0.05$, respectively; Figure 2.2h, i), indicating DT injury as potential cause of elevated urinary iron and transferrin excretion in patients with tubular dysfunction.

Urinary iron excretion correlated with tubular injury in patients with systemic iron overload

Finally, we examined if urinary iron excretion in systemic iron overload was associated with tubular injury. Patients with systemic iron overload showed increased urinary TBARs ($p<0.001$, Table 2.2). Whereas GSTP1-1 levels were not affected, urinary KIM-1 concentrations were increased in patients with systemic iron overload ($p<0.001$; Table 2.2), especially in patients with β-thalassemia major ($p<0.001$ compared to control, $p<0.05$ compared to HFE-HH; Table 2.3). Furthermore, urinary transferrin levels in β-thalassemia major correlated strongly with urinary KIM-1 levels ($r=0.71$, $p<0.05$; Figure 2.3), which might indicate reduced transferrin reabsorption as a consequence of PT injury.

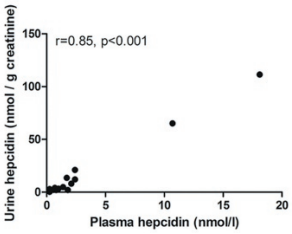

Figure 2.4: Urinary hepcidin correlates with plasma hepcidin concentrations in systemic iron overload
Correlation between urinary hepcidin and plasma hepcidin in patients with systemic iron overload (n=20). Each dot represents one patient. Presented with Spearman's correlation coefficient (r) and significance (p-value).

Urinary hepcidin excretion does not substantiate urinary iron excretion

In addition to plasma hepcidin levels, we also analyzed hepcidin excretion in urine, since urinary hepcidin was recently reported to protect against acute kidney injury.[40-42] We did not find an increase in urinary hepcidin levels in patients with tubular dysfunction (Table 2.2), and, therefore, we could not underline a role of hepcidin in these patients. In patients with systemic iron overload, urinary hepcidin concentrations were significantly reduced, especially in HFE-HH patients (Table 2.2, 2.3). Urinary hepcidin levels strongly correlated with plasma hepcidin concentrations ($r=0.85$, $p<0.001$; Figure 2.4), corroborating our previous findings.[43]

DISCUSSION

Increased urinary iron concentrations have been associated with renal injury. Here, we investigated the contribution of glomerular filtration of increased circulating iron and insufficient tubular iron reabsorption to urinary iron excretion in patients with either systemic iron overload or renal tubular dysfunction. Our results demonstrate that both increased circulating iron levels and subsequent filtration as well as insufficient tubular reabsorption are associated with increased urinary iron concentrations and, importantly, renal injury.

Patients with tubular dysfunction were included in this study to investigate the contribution of diminished PT TBI reabsorption to urinary iron concentrations. Although we cannot fully exclude the presence of glomerular injury in these patients, the high prevalence of proximal tubular dysfunction pathologies in our patient cohort together with our finding that increased urinary albumin excretion did not relate to eGFR, implicate that glomerular dysfunction are not likely to contribute to urinary iron and transferrin excretion in these patients. Recently, we reported iron deposition in PTs and DTs in

various CKD disorders.[44] These patients predominantly showed glomerular diseases, which would result in increased filtration of circulating iron levels into the tubular lumen and, subsequently, urinary iron excretion. In our cohort of patients with tubular dysfunction, we presume that iron filtration by the glomerulus and consequent iron levels reaching the tubular lumen is not affected. Although both the PT injury marker KIM-1 and the DT injury marker GSTP1-1 showed increased concentrations in urine, only GSTP1-1 correlated with urinary iron and transferrin concentrations. We propose that increased TBI levels reach the DT because of hampered PT reabsorption. Increased DT iron exposure could result in DT iron accumulation and subsequent DT injury.[5] In addition, our data suggest that DT iron reabsorption capacity is not sufficient to compensate for the increased iron delivery and, therefore, iron and transferrin are lost in urine in patients with tubular dysfunction. Therefore, both the PT and DT may contribute to urinary iron wasting during tubular dysfunction (Figure 2.5).

Mild systemic iron overload observed in our HFE-HH subjects did not result in urinary iron or NTBI excretion. Despite mild PT injury as indicated by the increased KIM-1 levels and oxidative stress as indicated by increased urinary TBARs, tubular iron reabsorption capacity was sufficient to prevent urinary iron excretion in patients with mild

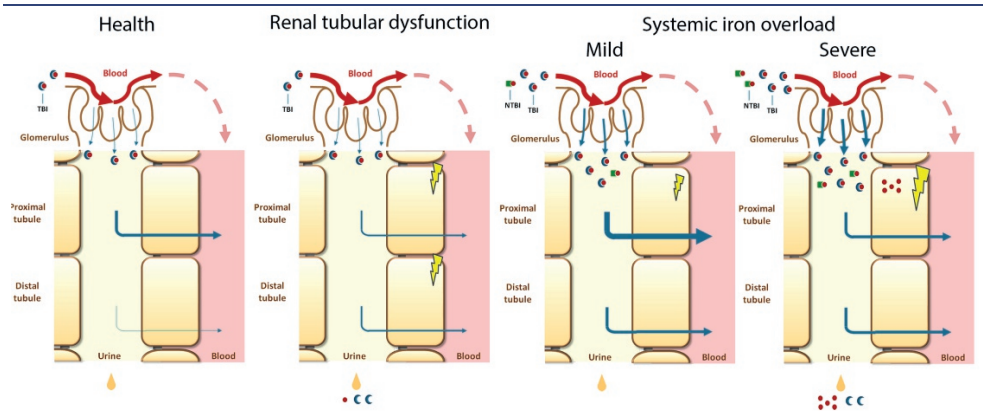

Figure 2.5: Proposed mechanism of iron reabsorption in the kidney in health, tubular dysfunction and systemic iron overload.

In health (left panel), circulating transferrin-bound iron (TBI) is filtered into the renal tubular lumen by the glomerulus, and subsequently is completely reabsorbed. This is predominantly done by proximal tubules (PTs) and only to a minor extent in distal tubules (DTs). During tubular dysfunction (second left panel), similar TBI levels are filtered into the tubular lumen, but these are reabsorbed to a lower extent by PTs as a result of PT injury (indicated by lightning sign). Consequently, DTs reabsorb larger amounts of TBI, but, as a result of DT injury, iron (in red) and transferrin (in blue) are excreted in urine. In mild systemic iron overload (second right panel), increased circulating TBI and non-transferrin-bound iron (NTBI) are filtered into the tubular lumen. Despite oxidative stress and mild PT injury, reabsorption in PTs and DTs prevents iron and transferrin excretion. In severe systemic iron overload (right panel), TBI and NTBI are filtered into the tubular lumen in even larger concentrations, but limitedly reabsorbed by PTs as a result of increased intracellular iron levels, oxidative stress and PT injury. Iron reabsorption capacity in DTs is not sufficient to prevent iron and transferrin excretion in urine.

iron overload (Figure 2.5). In contrast, patients with β-thalassemia major with severe iron overload, demonstrated increased urinary iron, transferrin and NTBI excretion, demonstrating urinary iron reabsorption capacity falls short in these patients. The difference in clinical presentation between these patients groups can be explained by the regular maintenance phlebotomy in HFE-HH patients, resulting in only mildly elevated TSAT, whereas ferritin levels were within the normal range. Nevertheless, severe iron overload has also been observed in patients with newly diagnosed HFE-HH patients.[18] Therefore, the extent of systemic iron overload, rather than the disease pathology, determines urinary iron excretion.

Besides glomerular filtration of increased circulating iron levels, disturbed tubular iron handling or tubular injury may also have contributed to the high urinary iron excretion observed in our β-thalassemia major subjects. Increased plasma ferritin levels are indicative of high tissue iron stores, including the kidney.[19] Moreover, mouse PT cells have been described to actively secrete ferritin proteins and ferritin is detectable in urine of healthy volunteers.[20,45] However, iron levels in secreted ferritin are low[45] and are, therefore, unlikely to largely contribute to urinary iron excretion. Alternatively, we cannot exclude that increased exfoliation of iron-loaded tubular epithelial cells contributed to urinary iron concentrations. Finally, PT injury observed in the β-thalassemia major patients may have influenced urinary iron and transferrin excretion. Presumably, PT injury limits PTTBI reabsorption leading to more TBI excretion that cannot be compensated for by DT reabsorption. The increased urinary TBAR concentrations suggest that PT injury in patients with β-thalassemia major may result from oxidative stress, potentially caused by increased urinary iron levels. However, we cannot rule out other causes of the observed renal injury. Altogether, urinary iron and transferrin excretion in β-thalassemia major may be resulting from filtration of increased circulating iron, enhanced iron levels in renal tubuli and/or (oxidative) PT injury (Figure 2.5).

Our finding of increased urinary KIM-1 levels in β-thalassemia major patients adds to the rising number of reports on PT injury in β-thalassemia,[46-49] and confirms previous suggestions that persistent severe systemic iron overload can lead to renal injury.[50] Tubular epithelial cells can be exposed to harmful levels of iron both apically from the tubular lumen and basolaterally from the systemic circulation. Furthermore, tubular injury may also be related to use of chelation mediation.[51-53] Future studies examining the etiology of renal injury during systemic iron overload are warranted, in order to prevent progressive kidney disease.

Our results showed a correlation between urinary hepcidin and circulating hepcidin levels, confirming our previous observations.[43] Although hepcidin has been reported to protect from acute kidney injury,[54,55] our results cannot confirm this.

Limitations of this study include the explorative and observational design with small subject groups. Although we aimed at including subjects in age- and gender-matched groups, patients with HFE-HH were older than patients with β-thalassemia major,

reflecting the average age of these patient groups in the population.[56,57] Furthermore, all study groups predominantly consisted of male subjects, except for the β-thalassemia major patients. Although age above 40 years and male gender have been reported to increase levels of urinary KIM-1 and albumin in healthy individuals,[58,59] this is unlikely to have largely influenced KIM-1 and albumin levels in urine in β-thalassemia. In addition, partly because the specific disorders are rare, the renal tubular dysfunction patient group consisted of a heterogeneous collection of disorders.

The knowledge that both filtration of systemic iron levels and tubular iron reabsorption influence urinary iron excretion has several implications. In patients with iron overload disorders prevention or reduction of tubular iron reabsorption could be used to enhance urinary iron excretion and, thus, lower the systemic iron burden and potential renal injury. The latter is also important for patients with chronic kidney disease. Glomerular injury leads to increased protein filtration, including TBI, resulting in increased urinary iron excretion and renal iron loading.[3,9-13] In CKD animal models, tubular injury was associated with urinary iron excretion and reduction of circulating iron levels by using a low-iron diet or treatment with an iron chelator reduced renal tubular injury.[60,61] In CKD, preventing renal iron accumulation by reducing tubular reabsorption could be an interesting therapeutic option.

In conclusion, our results suggest that both glomerular filtration of increased circulating iron levels and insufficient tubular iron reabsorption could increase urinary iron excretion and cause renal injury.

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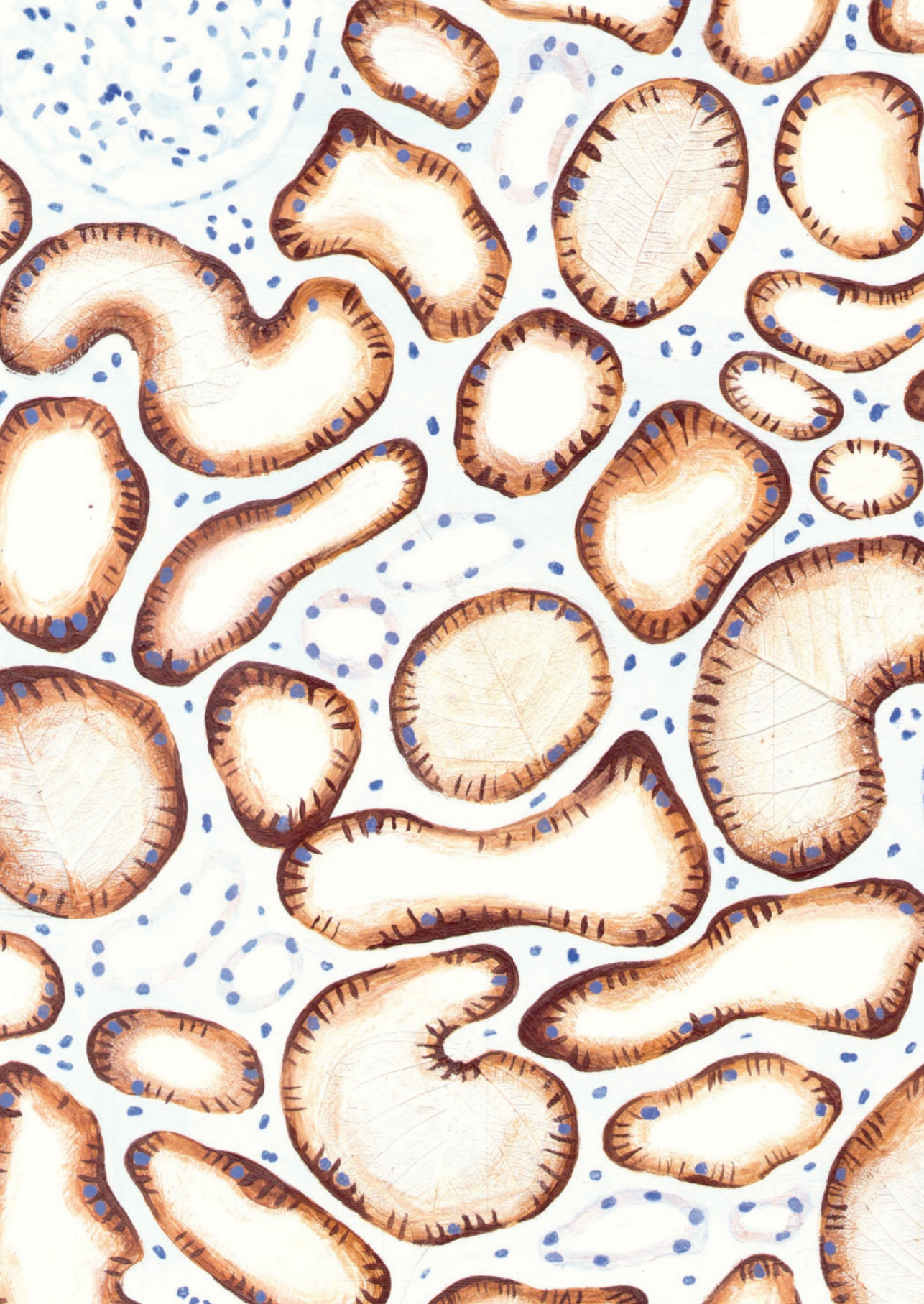

3

Iron uptake by ZIP8 and ZIP14 in human proximal tubular epithelial cells

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ABSTRACT

In patients with iron overload disorders, increasing number of reports of renal dysfunction and renal iron deposition support an association between increased iron exposure and renal injury. In systemic iron overload, elevated circulating levels of transferrin-bound (TBI) and non-transferrin-bound iron (NTBI) are filtered to the renal proximal tubules, where they may cause injury. However, the mechanisms of tubular iron handling remain elusive. To unravel molecular renal proximal tubular NTBI and TBI handling, human conditionally immortalized proximal tubular epithelial cells (ciPTECs) were incubated with ^{55}Fe as NTBI and fluorescently labeled holo-transferrin as TBI. Ferrous iron importers ZIP8 and ZIP14 were localized in the ciPTEC plasma membrane. Whereas silencing of either ZIP8 or ZIP14 alone did not affect ^{55}Fe uptake, combined silencing significantly reduced ^{55}Fe uptake compared to control ($p<0.05$). Furthermore, transferrin receptor 1 (TfR1) and ZIP14, but not ZIP8, colocalized with early endosome antigen 1 (EEA1). TfR1 and ZIP14 also colocalized with uptake of fluorescently labeled transferrin. Furthermore, ZIP14 silencing decreased ^{55}Fe uptake after ^{55}Fe-Transferrin exposure ($p<0.05$), suggesting ZIP14 could be involved in early endosomal transport of TBI-derived iron into the cytosol. Our data suggest that human proximal tubular epithelial cells take up TBI and NTBI, where ZIP8 and ZIP14 are both involved in NTBI uptake, but ZIP14, not ZIP8, mediates TBI-derived iron uptake. This knowledge provides more insights in the mechanisms of renal iron handling and suggests that ZIP8 and ZIP14 could be potential targets for limiting renal iron reabsorption and enhancing urinary iron excretion in systemic iron overload disorders.

INTRODUCTION

Iron is an essential element for life, but it can also be harmful by catalyzing the formation of reactive oxygen species in the Fenton reaction.[1] The human body is able to regulate iron uptake and storage, but has limited abilities to regulate iron excretion.[2] Therefore, disturbed intestinal iron uptake in hereditary hemochromatosis, and frequent red blood cell transfusions in β-thalassemia major, can result in systemic iron overload and organ damage.[2] In these disorders, patients present with increased levels of iron bound to the circulating transport protein transferrin (transferrin-bound iron; TBI).[2] Once transferrin is largely saturated, non-transferrin-bound iron (NTBI) can be detected.[3,4] In contrast to iron bound to transferrin, iron in NTBI is only loosely bound to molecules such as citrate, available for redox cycling and, therefore, considered a toxic iron species.[3,5] Nowadays, iron-removal therapies decrease hepatic and cardiac iron loading mortality and extend patient's lives.[6-9] However, in recent years, kidney function abnormalities have been arising in patients with β-thalassemia major,[10] including increased urinary excretion of N-acetyl-D-glucosaminidase (NAG) and β-2-microglobulin, indicators of renal proximal tubular damage.[11-16] Also, renal iron deposition has been observed in adult patients with hereditary hemochromatosis[17-21] or β-thalassemia syndromes.[14,22,23] *In vitro* studies have shown that iron exposure can result in decreased cellular viability in murine and human renal tubular epithelial cells.[24-26] Altogether, these findings support an association between increased renal tubular iron exposure and renal tubular injury in systemic iron overload.

Tubular epithelial cells in the kidney share many iron handling proteins with other organ systems, such as transferrin receptor 1 (TfR1), divalent metal transporters ZIP8 (SLC39A8), ZIP14 (SLC39A14) and divalent metal transporter 1 (DMT1, SLC11A2), and iron exporter ferroportin, but their precise cellular localization and function in renal iron handling remain uncertain.[27-29] Circulating TBI is suggested to be filtered into the tubular lumen by the glomerulus.[30,31] Subsequently, iron in the tubular lumen has been reported to be completely reabsorbed by endocytic transport in renal tubular cells.[28] In agreement, hardly any iron is found in urine of healthy volunteers.[32,33] TBI reabsorption has been reported to mainly take place in proximal tubular epithelial cells (PTs), facilitated by TfR1 and the megalin-cubilin receptor complex.[30,31,34,35] After dissociation from transferrin, iron transport from the endosome into the cytosol is suggested to involve a divalent metal transporter. Although not yet clarified in PTs, studies in other cells suggest a role for ZIP8, ZIP14 and/or DMT1 in this process.[36-38] In the cytosol, iron is oxidized by H-ferritin and stored in L-ferritin, may be utilized by iron requiring processes or exported into the blood stream by the cellular exporter ferroportin.[28] Tubular NTBI could be directly derived from the circulation by glomerular filtration or dissociated from filtered TBI as a result of acidification of the filtrate passing along the nephron.[27,28,39] *In vitro* studies have reported that ZIP8 and ZIP14 are involved in direct NTBI uptake from the plasma membrane,[37,40-43] but evidence for PTs is lacking.

In this study, we first characterized TBI and NTBI handling in human conditionally immortalized proximal tubular epithelial cells (ciPTECs). Human ciPTECs, originating from renal tissue, have previously been shown to express functional influx and efflux transporters for solute reabsorption and drug excretion.[44,45] Next, we examined the localization of divalent metal transporters ZIP8, ZIP14 and DMT1 in these cells, *i.e.* at the plasma membrane and/or in endosomes, and studied the role of ZIP8 and ZIP14 in mediating iron uptake from NTBI and TBI.

MATERIALS AND METHODS

Cell culture

ciPTECs (clone T1), kindly provided by the department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen,[44] were cultured using DMEM HAM's F-12 phenol red-free medium (Thermo Fisher Scientific) containing 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epithelial growth factor 40 pg/ml tri-iodothyronine (all Sigma Aldrich), 10% (v/v) fetal calf serum (FCS; Greiner Bio-one), and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific). Cells were cultured at 33°C and 5% CO_2 and maturated for 24h at 33°C and 5% CO_2 and 7 days at 37°C and 5% CO_2 prior to experiments. Cells were cultured on transwell permeable supports to obtain polarization (Corning® Transwell®, coated with 50 µg/ml collagen VI (both Sigma Aldrich)) for ZIP8, ZIP14, DMT1 and ferroportin immunostaining, ^{55}Fe transport and Alexa Fluor 546-conjugated human holotransferrin (Alexa546-Transferrin, Thermo Fisher Scientific) uptake studies. Transepithelial electrical resistance (TEER) measurements and FITC-inulin permeability analysis were performed as previously described.[44]

Iron exposure

To simulate NTBI exposure, cells were exposed to 0-500 µM ferric citrate (FeC, Sigma Aldrich). FeC was dissolved overnight in MQ water at 37°C and added to medium devoid of FCS. Cell pellets were collected and stored at -80°C prior to iron assessment or immunoblotting. For TBI uptake studies, cells were depleted of transferrin by incubation in DMEM HAM's F-12 medium without supplements for 2h and exposed to 25 µg/ml Alexa546-Transferrin for 30 min. In iron loading conditions, cells were instead exposed to 100 µM FeC and 100 µg/ml holo-transferrin (Sigma Aldrich) for 2h and subsequently exposed to Alexa546-Transferrin. Afterwards, cells were used for immunostaining as

described below. To simulate iron overload exposure, cells were exposed to 100 µM FeC in medium supplemented with FCS for 48h and used for immunostaining of ZIP8 and ZIP14.

Small interfering RNAs (siRNAs)

Cells were seeded at 20% confluency and transfected on two consecutive days with a 50 pmol (ZIP8, ZIP14) or 100 pmol (ferroportin) small interfering RNAs (siRNAs) (ON-TARGETplus SMARTpool siRNAs for SLC39A8, SLC39A14, SLC40A1 and Non-targeting pool as scrambled control, Dharmacon) and 5 µl oligofectamine in Opti-MEM (both Thermo Fisher Scientific). After 4h, fresh medium was added. Cells were analyzed 48h after the second transfection.

Protein isolation and immunoblotting

Proteins were isolated using RIPA buffer (0.15 M NaCl, 0.012 M Sodium Deoxycholate, 1% NP40, 0.1% SDS, 0.05 M Tris, pH 7.5, freshly supplemented with protease inhibitors (Roche Complete Mini, Roche)). Protein concentration was determined using the Pierce BCA assay kit according to the manufacturer's instructions (Thermo Fisher Scientific). Protein samples were prepared in loading buffer, separated on SDS-PAGE gels, transferred to a nitrocellulose or PVDF membrane and incubated with primary antibody overnight at 4°C. After 1h incubation at RT with secondary antibody, proteins were visualized on an Odyssey fluorescence scanner (total ferritin, β-actin) or LAS-3000 scanner for chemiluminescence (all other primary antibodies). Antibodies and dilutions are summarized in Supplementary Table 3.1.

Cell surface biotinylation

Cells were biotinylated with 0.5 mg/ml Sulfo-NHS-LC-LC-biotin (Thermo Fisher Scientific) for 30 min at 4°C with gentle shaking. Protein lysates were collected by scraping and incubated overnight with Neutravidin beads (Thermo Fisher Scientific) to isolate cell surface biotinylated proteins. After eluting cell surface proteins from the beads in Laemmli buffer (Biorad) supplemented with 50 mM DTT for 30 min at 37°C, proteins were directly used for immunoblotting since the Pierce BCA assay could not be applied in this buffer solution. Therefore, both membrane protein and total lysate protein fraction were loaded on SDS-PAGE gels to obtain comparable Na K ATPase bands and allow comparison of proteins of interest between the two fractions.

RNA isolation and quantitative PCR

RNA isolation was performed using TRIzol (Thermo Fisher Scientific) according to the manufacturer's instructions. A reverse transcription reaction was performed with 1 µg

RNA, 4 µl first strand buffer, 1 µl dNTPs (12.5 mM), 2.04 µl random primers, 2 µl DDT, 1 µl M-MLV (all Thermo Scientific) and 0.5 µl RNAsin (Promega Corporation). The PCR cycle consisted of 10 min at 20 °C, 45 min at 42°C and 10 min at 95°C. Quantitative PCR was performed on a CFX96 (Bio-rad) using 4 µl cDNA (10 ng/ml), 10 µl SYBR Green Power master mix (Applied Biosystems) and 6 µl primer mix (containing 1 µM forward primer and reverse primer). The PCR protocol was as follows: 7 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C, and 10 min at 95°C, with a measurement at the end of each cycle. Fold change values were calculated using the ΔΔCt formula. Primers are summarized in Supplementary Table 3.2.

Immunofluorescent staining

Cells seeded on coverslips or transwell supports were fixed with 4% paraformaldehyde or 2% paraformaldehyde supplemented with 4% sucrose, permeabilized with 0.5% Triton X-100, 0.2% Tween-20 or 1% SDS and incubated with primary antibody overnight at 4°C. Subsequently, cells were stained with fluorescent secondary antibody for 1h at RT and counterstained with DAPI (4',6-diamidino-2-phenylindole, 300 µM, Thermo Fisher Scientific). Images were taken using the Zeiss S/N 3834004266 or confocal Olympus FV1000. Co-stainings were performed for TfR1, ZIP8, ZIP14 and DMT1 with early endosome antigen 1 (EEA1). Antibodies are summarized in Supplementary Table 3.1.

Iron assessment

Intracellular iron level were determined using the chromogen bathophenanthroline as described.[46] Iron concentrations were calculated by comparison to a standard curve of ferrous sulphate and corrected for protein concentration.

^{55}Fe transport

Cells were incubated with 0.2 µM $^{55}FeCl_3$ (Perkin Elmer) in a mixture with 100 µM FeC and 1 mM ascorbic acid (Sigma Aldrich) in Krebs-Henseleit-HEPES (KH-H) buffer supplemented with 2.5 mM $CaCl_2 \cdot 2H_2O$, 25 mM $NaHCO_3$ and 10 mM HEPES pH 7.4 (Sigma Aldrich). For cells grown in transwell supports, cells were depleted of iron in DMEM HAM's F-12 medium without supplements for 2h and incubated with ^{55}Fe in KH-H in the apical compartment or in medium in the basolateral compartment for 8h at 37°C. For ZIP8 and ZIP14 siRNA experiments, cells were depleted of iron in KH-H buffer for 2h and incubated with ^{55}Fe for 30 min afterwards. For ferroportin siRNAs, cells were exposed to ^{55}Fe for 2h. Subsequently, cells were washed with ice-cold KH-H and harvested using RIPA buffer. Afterwards, radioactivity was measured using liquid scintillation counting. Radioactivity in protein pellets was corrected for protein concentration.

^{55}Fe-TBI was prepared by incubating apo-transferrin (Sigma Aldrich) with 7.5 nmol $^{55}FeCl_3$ and 75 nmol Sodium Citrate in KH-H for 1h at RT. Unbound iron was removed by repeated washing in 30K Amicon filter units. After iron depletion in KH-H buffer for 2h, cells were exposed to 2 uM ^{55}Fe-TBI for 4h and analyzed as described above.

Statistical analysis

Data were statistically analyzed using GraphPad Prism and presented as mean ± SEM. Results were analyzed by one-way ANOVA with Dunnett's post test or Student's t-test where appropriate. Differences were considered statistically significant when $p<0.05$.

RESULTS

Characterization ciPTEC model for studying iron handling

We first characterized the presence and abundance of known iron handling proteins in ciPTECs in unstimulated conditions and after iron exposure. Upon exposure to NTBI (FeC), ciPTECs showed a time-dependent increase in intracellular iron levels, which was statistically significant for 100 and 200 µM FeC after exposure for 16 and 24h (Figure 3.1a). This was complemented by increased total ferritin and decreased TfR1 protein levels compared to control (Figure 3.1b), confirming genomic iron responsive element – iron responsive protein (IRE-IRP) regulation of these proteins in ciPTECs.[47] Next, we examined iron uptake in polarized ciPTECs, grown on transwell supports. Monolater integrity was confirmed by minimal paracellular permeability of the diffusion marker FITC-inulin (7.6 ± 0.8 pmol/min/cm^2), appropriate TEER (≥140 Ω/cm^2) and clear expression of the tight junction protein zona occludens 1 (ZO-1; Figure 3.1c).[44] In ciPTECs cultured on transwell supports, apical ^{55}Fe exposure resulted in intracellular iron loading while basolateral ^{55}Fe exposure showed only limited cellular uptake (Figure 3.1d). This indicates that ciPTECs take up iron mainly from the apical cellular side. Additionally, we characterized the direction of iron export in ciPTECs. Polarized ciPTECs showed basolateral iron export after apical ^{55}Fe exposure, while basolateral ^{55}Fe exposure showed negligible apical iron export (Figure 3.1d). CiPTECs also demonstrated uptake of Alexa546-Transferrin (holo-transferrin), which was diminished in combination with unlabelled holo-transferrin, indicating ligand-specific competition, or at 4°C, suggesting active uptake (Figure 3.1e). Alexa546-Transferrin uptake was mostly observed after apical exposure, and little uptake was seen after basolateral exposure, whereas both apical and basolateral uptake was reduced in iron-loaded cells (Figure 3.1f). The latter suggests that ciPTECs regulate TBI uptake based on intracellular iron levels through IRE-IRP regulation.

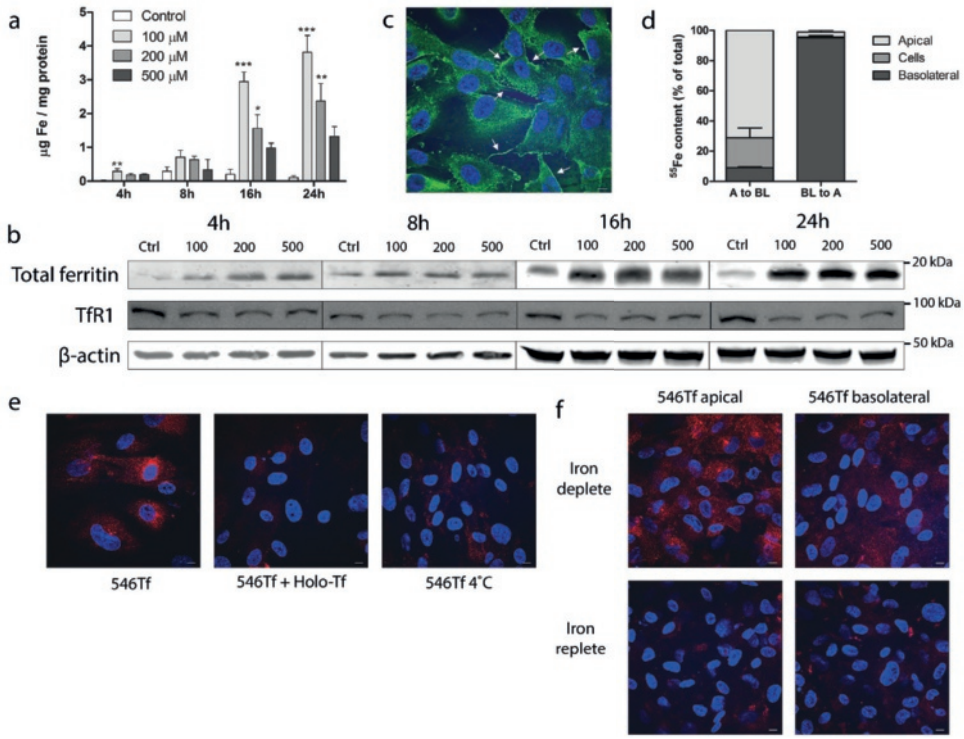

Figure 3.1 Uptake of non-transferrin-bound iron and transferrin-bound iron in ciPTECs

Intracellular iron concentration (a), total ferritin and transferrin receptor 1 (TfR1) protein levels (b) in ciPTECs after incubation with different concentrations of ferric citrate (NTBI). Zona occludens 1 (ZO-1) immunostaining (in green) confirming monolayer integrity in polarized ciPTECs. ZO-1 tight junctions indicated by arrows (c). ^{55}Fe content in apical compartment, basolateral compartment and cell lysate after ^{55}Fe exposure from the apical (A to BL) or basolateral (BL to A) cellular side (d). Alexa546-Transferrin (546Tf, in red) internalization alone, combined with holo-transferrin (Holo-Tf) or at 4°C (e). 546Tf internalization in iron deplete or iron replete conditions, from apical or basolateral cellular side (f). Nuclei counterstained with DAPI (in blue). Representative images and graphs showing mean of three independent experiments for each time point or FeC concentration except n=6 for panel c. Scale bar 5 µM. One-way ANOVA with Dunnett's post test compared to control at each time point was used in a; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

We further investigated the role of ferroportin in iron export observed in ciPTECs. Ferroportin immunostaining showed localization near the basolateral cellular side (Figure 3.2a), in agreement with its presumed role in iron export in PTs to the plasma.[48] To study the functional contribution of ferroportin to iron export, we used siRNA technology. Successful ferroportin knockdown was confirmed on mRNA level ($p<0.001$, Figure 3.2b). Ferroportin knockdown increased cellular ^{55}Fe content compared to scrambled control, while ^{55}Fe in the exposure solution was decreased ($p<0.05$ and $p<0.01$, respectively, Figure 3.2c). This was complemented by increased total ferritin protein levels and a trend of decreased *TfR1* mRNA levels in ferroportin knockdown compared to control (Figure 3.2d).

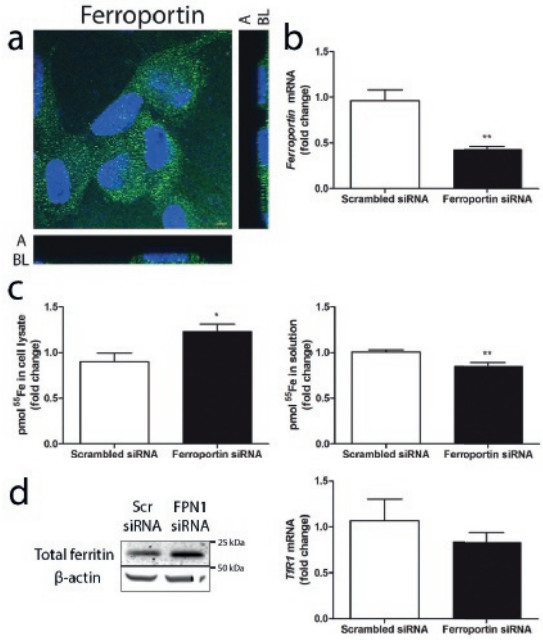

Figure 3.2 Ferroportin-mediated iron export in ciPTECs

Ferroportin immunostaining (in green) in polarized ciPTECs (a). Nuclei counterstained with DAPI (in blue). Confocal images taken in x-y and y-z axis showing apical (A) and basolateral (BL) cellular side. CiPTEC *ferroportin* mRNA expression (b), pmol ^{55}Fe in cell lysate or solution (c), total ferritin protein expression and *transferrin receptor 1 (TfR1)* mRNA expression (d) after transfection with scrambled control (Scr) or ferroportin (FPN1) small interfering RNAs (siRNA). Representative images and graphs showing mean of at least three independent experiments (a, n=3; b, n=9; c, n=4; d, both n=3). Scale bar 5 µM. Student's t-test was used in b, c and d; * $p<0.05$; ** $p<0.01$.

These data confirm that ferroportin functions as an iron exporter in ciPTECs at the basolateral membrane.

NTBI uptake involves ZIP8 and ZIP14

To investigate whether ZIP8 and ZIP14 could mediate apical NTBI uptake, we examined their localization in ciPTECs. We found ZIP8 and ZIP14 immunostaining near the apical cellular side in ciPTECs cultured on transwell supports (Figure 3.3a). We applied cell surface biotinylation and subsequent immunoblotting to investigate the localisation of these transporters at the plasma membrane and/or in intracellular endosomes. Succesful membrane isolation was confirmed by the presence of plasma membrane marker Na K ATPase in the cell surface fractions, whereas the endosomal marker EEA1 and cytosolic protein β-actin were absent compared to the total cell fractions (Figure 3.3b). We detected both ZIP8 and ZIP14 in the ciPTEC plasma membrane and total cell lysate fraction. In contrast, DMT1 immunostaining was detected near the apical membrane, but

we did not detect this protein in cell surface fractions (Figure 3.3a, b). This indicates ZIP8 and ZIP14 are potential candidates for NTBI uptake at the plasma membrane and subsequent experiments have, therefore, focused on ZIP8 and ZIP14.

We applied ZIP8 and ZIP14 siRNA technology to knockdown each transporter on mRNA level (60% *ZIP8* and 60% *ZIP14* mRNA remaining compared to scrambled control; $p<0.001$), without affecting mRNA expression of the other ZIP transporter (*ZIP14* and *ZIP8*, respectively) (Figure 3.4a, b). Protein expression was decreased to 50% of scrambled control for ZIP8 and 60% for ZIP14 (Figure 4a, b). However, silencing of either ZIP8 or ZIP14 did not significantly reduce ^{55}Fe uptake (85% and 95% of scrambled control,

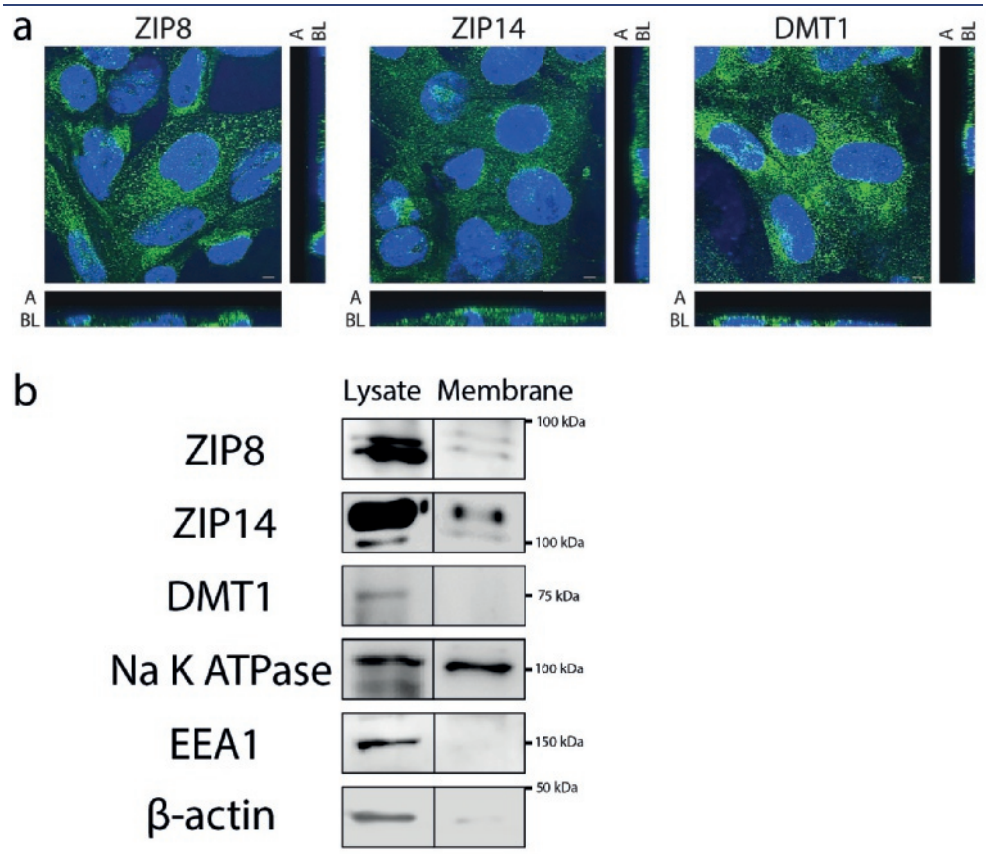

Figure 3.3 Presence of ZIP8, ZIP14 and DMT1 in ciPTECs

ZIP8, ZIP14 and divalent metal transporter 1 (DMT1) immunostaining (in green) in polarized ciPTECs. Nuclei counterstained with DAPI (in blue). Confocal images taken in x-y and y-z axis showing apical (A) and basolateral (BL) cellular side (a). Cell surface biotinylation and immunoblotting of ZIP8, ZIP14 and DMT1 in both membrane fraction and total lysate fraction. In addition, Na K ATPase was used as positive control for cellular membrane proteins, early endosome antigen 1 (EEA1) as negative control for endosomal proteins and β-actin as negative control for cytosolic proteins. Similar Na K ATPase protein levels were loaded in both membrane fraction and total lysate fraction. Prolonged chemiluminescence confirmed depicted findings (data not shown) (b). Representative images showing three independent experiments. Scale bar 5 μM.

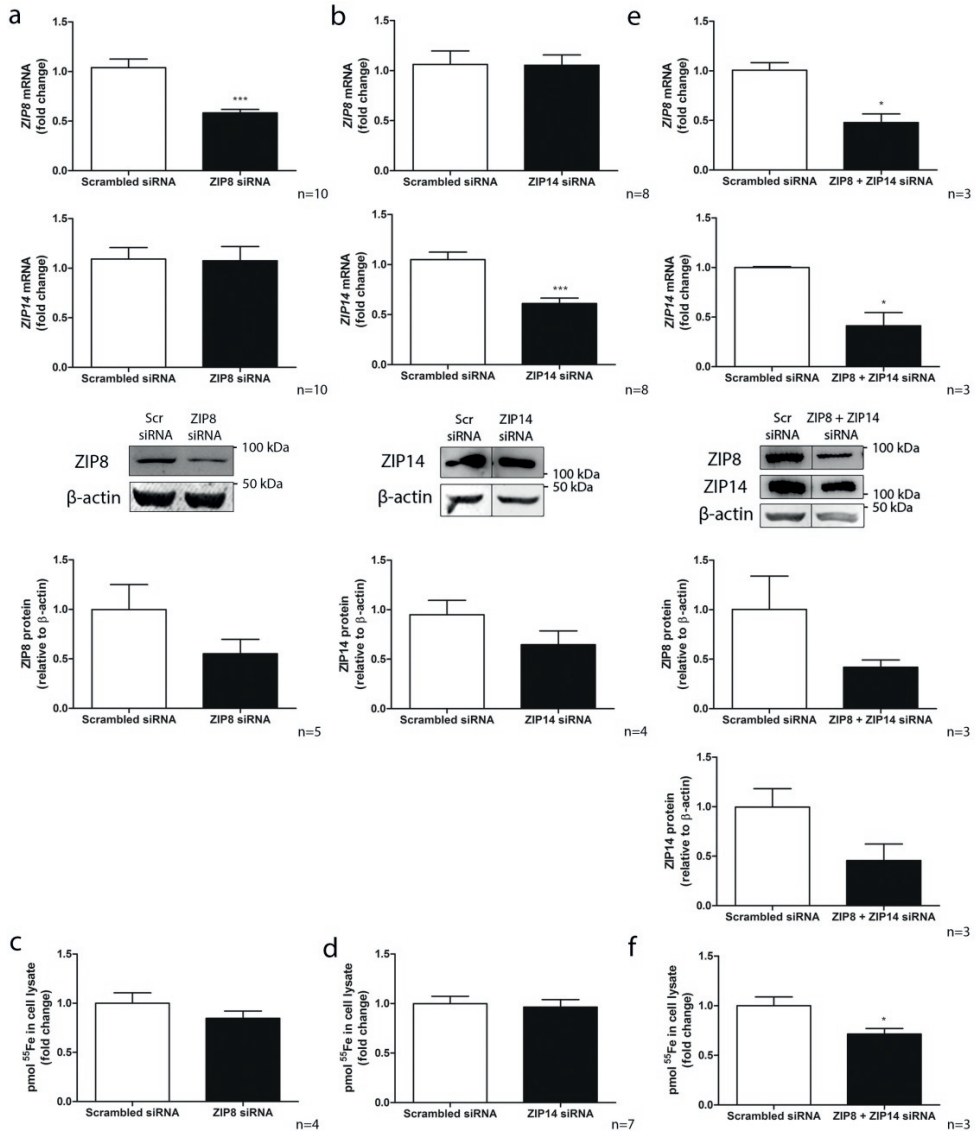

Figure 3.4 ZIP8 and ZIP14 mediate NTBI uptake in ciPTECs

ZIP8 and *ZIP14* mRNA and protein expression after transfection with scrambled control (Scr) and either ZIP8 (a) or ZIP14 small interfering RNA (siRNA) (b). Pmol ^{55}Fe uptake in cell lysate after either ZIP8 (c) or ZIP14 siRNA transfection (d). *ZIP8* and *ZIP14* mRNA and protein expression (e) and pmol ^{55}Fe uptake in cell lysate (f) after transfection with scrambled control or combined ZIP8 + ZIP14 siRNAs. Representative graphs. Number of experiments depicted in each panel. Student's t-test was used in a-e; * $p<0.05$; *** $p<0.001$.

respectively, Figure 3.4c, d). Since both transporters are described to have similar iron transport capacities,[49] we also applied combined siRNA knockdown. Both transporters were successfully downregulated on mRNA and protein level (50% *ZIP8* and 40% *ZIP14*

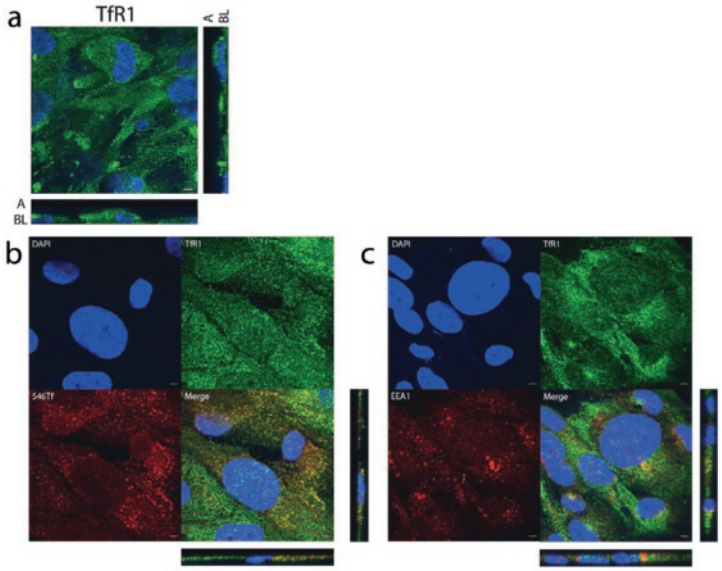

Figure 3.5 Transferrin receptor 1 mediates Alexa546-Transferrin uptake in ciPTECs
Transferrin receptor 1 (TfR1) immunostaining (in green) in polarized ciPTECs (a). TfR1 immunostaining (in green) colocalization with Alexa546-Transferrin (546Tf) internalization (in red) (b) or early endosome antigen 1 (EEA1) immunostaining (in red) (c). Nuclei counterstained with DAPI (in blue). Confocal images taken in x-y and y-z axis showing apical (A) and basolateral (BL) cellular side. Representative images of three experiments. Scale bar 5 µM.

mRNA, and 50% ZIP8 and 50% ZIP14 protein remaining, respectively; Figure 3.4e). Knockdown of both transporters significantly reduced ^{55}Fe uptake (70% of control, $p<0.05$, Figure 3.4f). This indicates that both ZIP8 and ZIP14 play a role in NTBI uptake and indeed show redundancy in ciPTECs.

TBI-derived iron uptake involves ZIP14

We detected TfR1 near the ciPTEC apical membrane using fluorescent immunostaining (Figure 3.5a) and found TfR1 to colocalize with both EEA1 and Alexa546-Transferrin (Figure 3.5b, c), indicating TBI uptake involves TfR1 via endocytosis. Subsequently, we examined whether ZIP8 and ZIP14 may play a role in iron uptake resulting from TBI endocytosis. We found only ZIP14,but not ZIP8, to colocalize with EEA1 (Figure 3.6a), suggesting a possible involvement of ZIP14 in transport of iron out of the endosome towards the cytosol. This staining pattern was not affected by iron exposure (Figure 3.6b), indicating ZIP8 is absent from ciPTEC endosomes independent of cellular iron status. ZIP14 immunostaining colocalized with Alexa546-Transferrin uptake (Figure 3.6c), but ZIP14 knockdown did not affect Alexa546-Transferrin uptake (Figure 3.6d). Cellular ^{55}Fe content, however, was decreased in ZIP14 knockdown cells compared to control after ^{55}Fe-TBI exposure ($p<0.05$, Figure 3.6e). This indicates ZIP14 could be involved in TBI-derived iron uptake. DMT1 was also found to colocalize with EEA1 immunostaining (Figure 3.6f) and,

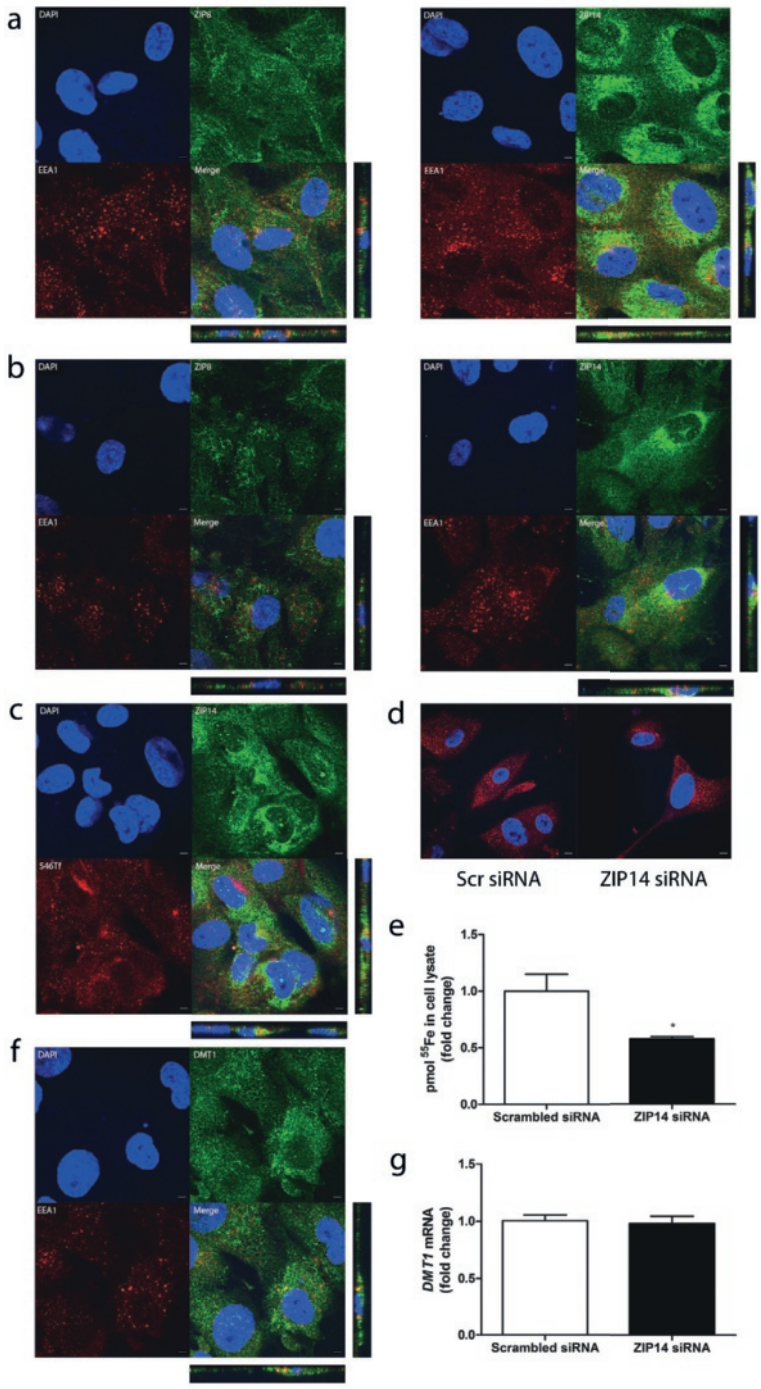

Figure 3.6 ZIP14 mediates Alexa546-Transferrin uptake in ciPTECs

Double immunostaining of ZIP8 or ZIP14 (in green) and early endosome antigen 1 (EEA1, in red) in unstimulated conditions (a) or after 48h iron overload exposure (b). ZIP14 immunostaining (in green) colocalization with Alexa546-Transferrin (546Tf) internalization (in red) (c). 546Tf internalization (d) or pmol ^{55}Fe in cell lysate after ^{55}Fe-TBI exposure (e) after transfection with scrambled control or ZIP14 small interfering RNA (siRNA). Double immunostaining of divalent metal transporter 1 (DMT1, in green) and EEA1 (in red) (f). *DMT1* mRNA expression (g) after transfection with ZIP14 or scrambled control siRNAs. Nuclei counterstained with DAPI (in blue). Confocal images show x-y and y-z axis. Representative images and graphs of three experiments. Scale bar 5 µM. Student's t-test was used in e, g; * $p<0.05$.

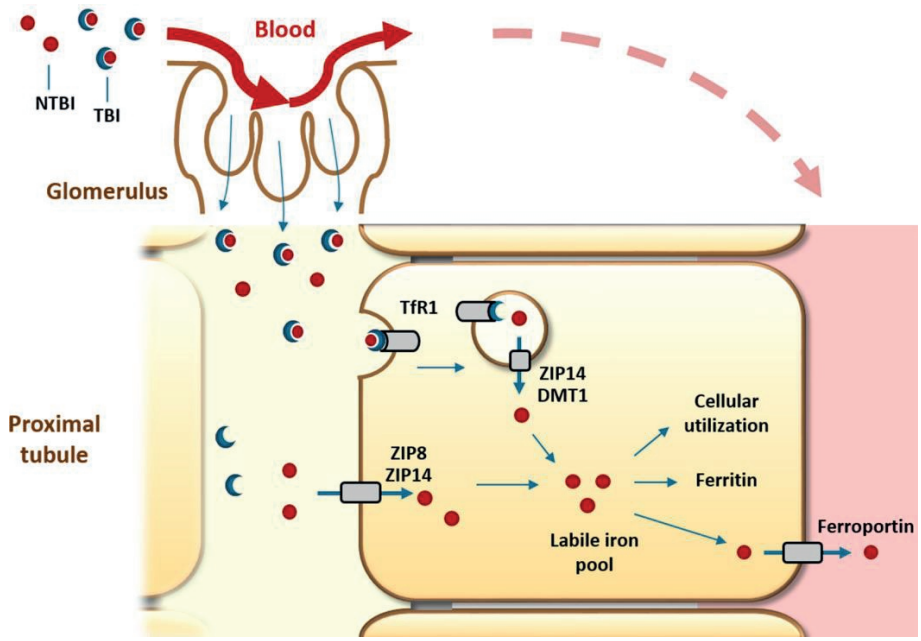

Figure 3.7 Proposed NTBI and TBI handling in proximal tubular epithelial cells

Non-transferrin-bound iron (NTBI) and transferrin-bound iron (TBI) present in the systemic circulation are filtered by the glomerulus into the tubular lumen and are subsequently reabsorbed by proximal tubular cells. NTBI uptake at the plasma membrane involves both ZIP8 and ZIP14 that show redundancy. TBI uptake involves TfR1-mediated endocytosis. Subsequently, iron is transported from the endosome towards the cytosol via ZIP14 and potentially also divalent metal transporter 1 (DMT1). Once in the cytosol, in the labile iron pool, iron is utilized, stored in ferritin or exported back into the circulation by ferroportin at the basolateral membrane.

even though *DMT1* mRNA expression did not change with ZIP14 knockdown (Figure 3.6g), ZIP14 and DMT1 could be redundant in TBI-derived iron uptake.

DISCUSSION

Evidence of renal complications in patients with systemic iron overload is accumulating, while the molecular mechanisms of renal NTBI and TBI handling in systemic iron overload remain unclear. We have shown that human PTs take up both NTBI and TBI. NTBI uptake involves both ZIP8 and ZIP14, which demonstrate redundancy. In contrast, TBI uptake is mediated by TfR1 endocytosis, where ZIP14, but not ZIP8, may be involved in iron release from the endosome into the cytosol (Figure 3.7).

In addition to studies that report the suitability of ciPTECs to study renal physiology,[44,45] we have demonstrated that this model is also qualified to study renal PT iron handling as it contains mechanisms for iron uptake, intracellular handling and export. Using this model, we newly demonstrated the subcellular distribution of endogenous ZIP8 and ZIP14 in human PTs using cell surface biotinylation, immunoblotting and

immunofluorescent colocalization stainings, which extends previous studies that mostly relied on overexpression models.[28] Our results show variability in divalent iron transporter localization in PT cells in terms of their localization at the plasma membrane or at the endosomal membrane. NTBI exposure was experimentally approached by addition of FeC to serum-free medium, preventing TBI formation, but allowing formation of citrate- bound NTBI. Interestingly, we observed a difference in involvement of ZIP8 and ZIP14 in NTBI and TBI uptake by ciPTECs. Although siRNA silencing in ciPTECs was limited, *i.e.* silencing of ~50% was achieved for all examined proteins, including ZIP8, ZIP14, ferroportin and ZIP8 and ZIP14 combined, it nevertheless demonstrated that both ZIP8and ZIP14 were involved in NTBI uptake, whereas only ZIP14 may play a role in TBI-derived iron uptake. These observations support the notion that we studied uptake of two distinct iron species. ZIP8 and ZIP14 were both present at the ciPTEC plasma membrane and showed redundancy in tubular NTBI import. This redundancy for plasma membrane NTBI uptake is likely since both ZIP8 and ZIP14 are reported to transport iron together with HCO_3^- at pH 6.5-7.5.[36,41,42,50] Also in hereditary hemochromatosis mouse models lacking ZIP14, NTBI uptake in the kidney was still detected, further corroborating redundancy of iron transporters.[51] Besides ZIP8 and ZIP14, other mechanisms for NTBI uptake have been described in various organs, which could explain why even the combined silencing of ZIP8 and ZIP14 did not abolish NTBI uptake in our model. In human hepatocytes, DMT1 was detected at the plasma membrane and was found to mediate NTBI uptake in these cells.[38] Although we previously detected DMT1 in human PTs,[52] it was not present at the plasma membrane in ciPTECs. As a result, we believed that a role for DMT1 in direct NTBI uptake at the plasma membrane was unlikely and focussed our studies on ZIP8 and ZIP14. Furthermore, in the circulation, NTBI can be bound to albumin[53] and, as such, bereabsorbed in the PT by the multiligand megalin-cubilin receptor complex.[54] Alternatively, L-type voltage-dependent calcium channels have been reported to facilitate NTBI uptake in cardiomyocytes,[55,56] which may also be the case in the human kidney, as these channels have been demonstrated in rat kidney.[57]

We showed that TBI is internalized predominantly from the apical cellular side and this is mediated by the endocytic transporter TfR1. In addition, also the megalin-cubilin receptor complex is reported to take up TBI in PTs.[35] The fact that TBI uptake was reduced during iron loading implies IRE-IRP-regulated restriction of TBI uptake, hence suggesting that TBI uptake in PTs is predominantly mediated through TfR1. Moreover, TfR1 reduction in iron excess conditions suggests that TBI uptake is not the major cause of PT iron accumulation during iron overload. Since ZIP8 and ZIP14 abundance is not controlled by IRE-IRP regulation,[49] it is plausible that especially unrestricted tubular NTBI uptake by these proteins results in renal iron loading and potentially subsequent iron-mediated kidney injury during systemic iron overload.

In ciPTECs, ZIP14 may play a role in transport of iron from the endosome towards the cytosol. In the endosome, ferric iron is released from transferrin, and, subsequently,

reduced to ferrous iron, possibly by a STEAP protein or Prion protein, which have been found in human and mouse kidney, respectively.[58-60] Iron is found to dissociate from transferrin at pH 6.5,[61] and ZIP14 is shown to transport iron at a similar pH.[36,41] Furthermore, ZIP14 is shown to mediate iron uptake from TBI in hepatocytes,[36] while both ZIP8 and ZIP14 are found to mediate iron uptake from TBI uptake in neurons.[37] Our results support the novel conclusion that ZIP14, but not ZIP8, may be involved in TBI-derived iron uptake in PT early endosomes. Our data do not substantiate the presence of ZIP8 in endosomes, which was shown for overexpressed rat ZIP8 in HEK293 cells.[42] However, TBI-derived iron uptake was not abolished with ZIP14 knockdown. Indeed, renal iron loading was observed in several hereditary hemochromatosis mouse models with TBI exposure despite ZIP14 knockout.[51] Besides ZIP14, we detected DMT1 in endosomes in ciPTECs and, therefore, DMT1 is a potential candidate for endocytic iron transport in PTs, like is reported in hepatocytes.[62-64] DMT1 is shown to transport iron at pH 5.5.[62] Therefore, it is possible that DMT1 transports iron into the cytosol from late endosomes, which are characterized by a pH between 4.5 and 5.5[41] in PTs, and this is an interesting topic for future studies (in ciPTECs). Moreover, also TRPML1 (mucolipin1) is reported to transport iron in late endosomes or early lysosomes.[65] The importance of this transporter in endosomal iron uptake is illustrated by findings of decreased cytosolic iron levels in TRPML1 knockout fibroblasts, and iron-deficiency and anaemia in patients with TRPML1 mutations.[65,66] TRPML1 has also been found in the kidney and could, thus, mediate endosomal iron transport into the cytosol.[67]

Although ZIP8 and ZIP14 demonstrated redundancy in ciPTEC NTBI transport, differential functions for both transporters have been reported. Despite similar iron transport capacities,[49] ZIP8 and ZIP14 show a different localization pattern at the organ level, suggesting that these transporters have different roles in NTBI uptake from the circulation. While ZIP14 has been reported as the main NTBI uptake transporter in the liver and pancreas,[51] ZIP8 is implicated to be the major NTBI transporter in hippocampal neurons.[37] As such, mutations in ZIP8 and ZIP14 in patients both lead to changes in metal transport, but show distinct phenotypes.[68-71] In addition to PTs, ZIP8 and ZIP14 are also present in human distal tubular epithelial cells,[52,72] but it remains to be investigated to what extent both transporters are involved in NTBI handling in the distal nephron.

In our current study in human ciPTECs, we observed ferroportin expression at the basolateral side of the cell, whereas expression at the apical and basolateral membrane was reported previously in murine PT.[39,73-76] We recently showed ferroportin expression in human kidney biopsies also at the basolateral cellular side.[52] Moreover, our findings in ciPTECs show that ferroportin functioned solely as iron exporter, and not as iron importer as has previously been suggested.[76] Our results are in line with the general belief of a ferroportin cellular iron export function.[77] Since to date no other iron cellular iron exporters have been identified, ferroportin is essential for physiological renal iron handling. Moreover, reabsorption of filtered iron and export back into the systemic

circulation is crucial for maintaining adequate body iron levels as well as preventing intracellular renal iron loading and potential iron-induced renal toxicity.

In summary, our findings in human ciPTECs show that ZIP8 and ZIP14 are involved in TBI and NTBI uptake. Nevertheless, the results also demonstrate high redundancy in divalent metal transport in ciPTECs, suggesting a complex mechanisms of iron uptake. Future studies should be aimed at elucidating the apparent complex process of proximal tubular iron reabsorption to further assess the contribution of individual transporters to iron uptake in physiological conditions and during systemic iron overload.

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SUPPLEMENTARY INFORMATION

Supplementary Table 3.1: Overview used primary antibodies

Protein	Primary antibody
TfR1	Thermo Fisher Scientific 136800, 1:500
ZIP8	ProteinTech 20459-1-AP, 1:500
ZIP14	Atlas Antibodies HPA016508, 1:500
DMT1	Abcam ab55735, 1:350
ZO-1	Invitrogen 402200, 1:100
EEA1	Abcam ab2900, 1:1000 (immunoblot and immunostaining with DMT1) Abcam ab70521, 1:200 (immunostaining with TfR1, ZIP8, ZIP14)
Na K ATPase	Koenderink lab, 1:2500[1]
Total ferritin	Sigma Aldrich F5012, 1:250
Ferroportin	Abcam ab85370, 1:100
β-actin	Sigma Aldrich A5441, 1:100,000

DMT1, divalent metal transporter 1; EEA1, early endosome antigen 1; TfR1, transferrin receptor 1; ZO-1, Zona occludens 1.

Supplementary Table 3.2: Overview used quantitative PCR primers

Protein	Forward primer (5'- 3')	Reverse primer (5'- 3')
TfR1	CCCAGTTGCTGTCCTGATATAGA	TTGAGAAAACAATGCAAAATGTG
ZIP8	TTATCTCTGTCCCCCTTTGTCCTC	CGCCTTCGAAAGAACAGCAG
ZIP14	CTGGACCACATGATTCCTCAG	GAGTAGCGGACACCTTCCAG
DMT1	ATGGCCCTCACATTTGGATA	CGAACATGCCCTTGAGTACC
Ferroportin	GGGGTCGCCTAGTGTCAT	CAGGTAGTCGGCCAAGGAT
HPRT	GCTATAAATTCTTTGCTGACCTGCTG	AATTACTTTTATGTCCCCTGTTGACTGG

DMT1, divalent metal transporter 1; HPRT, Hypoxanthine-guanine phorphoribosyltransferase; TfR1, transferrin receptor 1.

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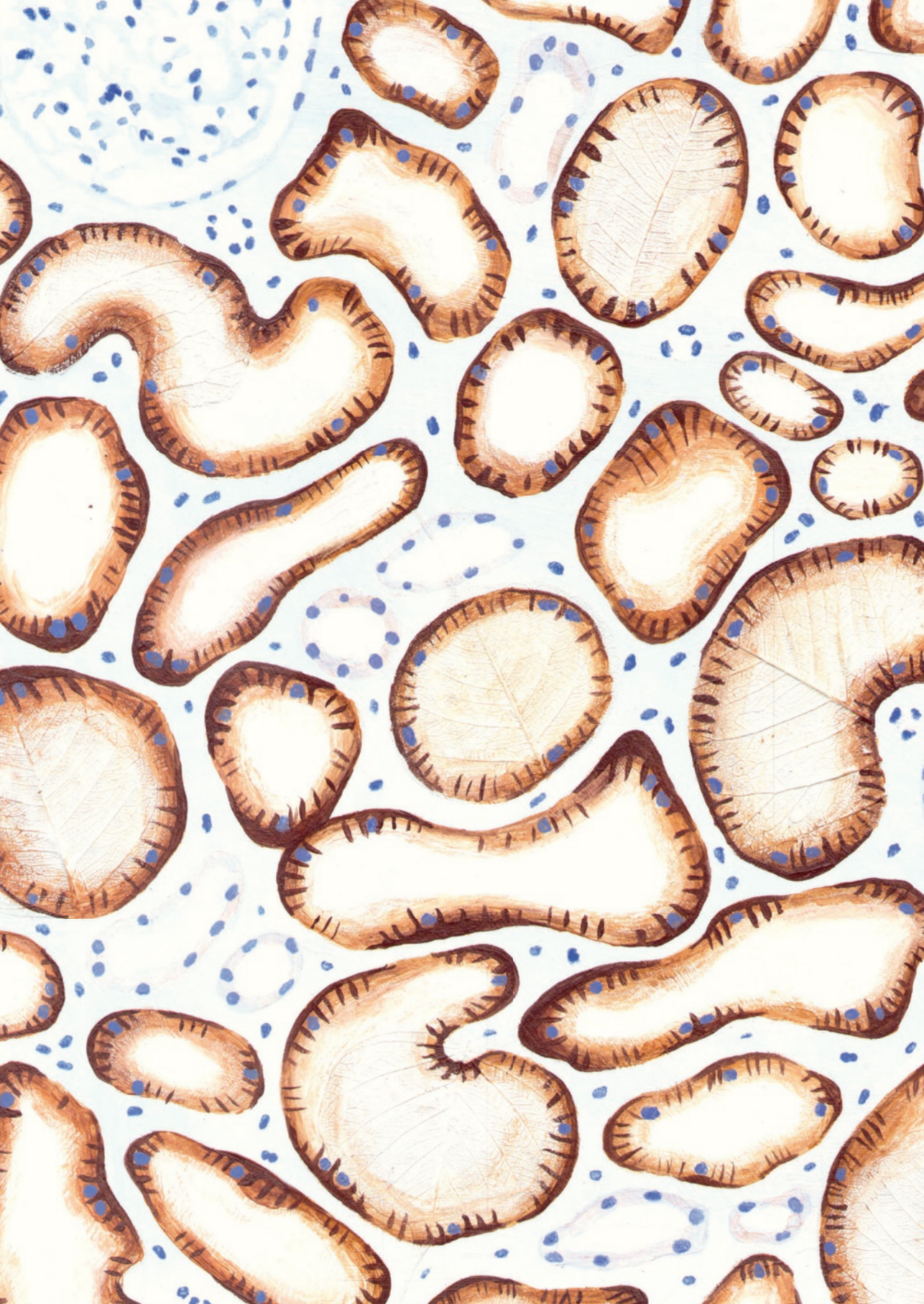

4

Inhibition of Nrf2 alters cell stress induced by chronic iron exposure in human proximal tubular epithelial cells

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ABSTRACT

Iron can catalyze reactive oxygen species (ROS) formation, causing cellular injury. In systemic iron overload, renal tubular epithelial cells are luminally exposed to high iron levels due to glomerular filtration of increased circulating iron. Reports of tubular dysfunction and iron deposition in β-thalassemia major support an association between increased chronic iron exposure and renal tubular injury. In acute iron exposure, Nuclear factor-erythroid 2-related factor 2 (Nrf2) may protect from iron-induced injury, whereas chronic renal stress may lead to Nrf2 exhaustion. We studied the cytotoxic mechanisms of chronic iron exposure using human conditionally immortalized proximal tubular epithelial cells (ciPTECs). Long-term iron exposure resulted in iron accumulation, cytosolic ROS formation and increased *heme oxygenase 1 (HMOX-1)* mRNA expression (all $p<0.001$). This was accompanied by nuclear translocation of Nrf2 and induction of its target protein NQO1, which both could be blocked by the Nrf2 inhibitor trigonelline. Interestingly, iron and trigonelline incubation reduced ROS production, but did not affect *HMOX-1* mRNA levels. Moreover, ferritin protein and *CHOP* mRNA expression were induced in combined iron and trigonelline incubated cells ($p<0.05$). Together, these findings suggest that chronic iron exposure induces oxidative stress and that exhaustion of the antioxidant Nrf2 pathway may lead to renal injury.

INTRODUCTION

Iron is indispensable for life, but it can also be harmful by catalyzing reactive oxygen species (ROS) formation in the Fenton reaction.[1] The human body carefully regulates iron uptake and storage, but has limited abilities to regulate iron excretion.[2] As a result, increased intestinal iron uptake in hereditary hemochromatosis and increased body iron acquisition through frequent red blood cell transfusions in β-thalassemia syndromes have been shown to result in chronic systemic iron overload and subsequent organ damage.[2] Patients with systemic iron overload suffer from elevated circulating iron levels, which are bound to the transport protein transferrin (transferrin-bound iron, TBI).[2] Once transferrin becomes largely saturated with iron, non-transferrin-bound iron (NTBI) can be detected.[3,4] Although iron is tightly bound to transferrin in TBI, iron is only loosely bound to small molecules such as citrate, in NTBI.[3] As such, iron in NTBI is available for redox cycling and is, therefore, considered a toxic iron species.[3,5] Circulating TBI and NTBI are filtered into the renal tubular lumen by the glomerulus.[6-8] Subsequently, iron in the tubular lumen is suggested to be completely reabsorbed by renal tubular cells, since hardly any iron is present in urine of healthy volunteers.[9,10] As a result, in patients with elevated circulating iron levels, proximal tubular epithelial cells (PTECs) are chronically exposed to high and potentially harmful iron levels.

In patients with hereditary hemochromatosis[11-15] or β-thalassemia major,[16,17] renal iron deposition has been observed. Moreover, renal dysfunction in patients with β-thalassemia major has been reported with increased urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) and β-2-microglobulin, both markers for renal PTEC damage.[16-21] Combined, these observations suggest that chronic iron overload may cause increased iron accumulation in the kidney, and, as such, may lead to clinically relevant nephrotoxicity over time.

The question remains, however, which exact molecular mechanisms are involved in renal tubular injury during chronic TBI and NTBI exposure. Previous animal and human studies suggest that chronically increased renal tubular iron exposure and injury in systemic iron overload depend on the balance between oxidative stress and antioxidative systems.[22-28] The major cellular pathway that protects against oxidative injury has been shown to be coordinated by Nuclear factor-erythroid 2-related factor 2 (Nrf2),[29] which is reported to protect from short term iron-induced injury in PTECs. Nrf2 knockout mice showed increased PTEC injury and urinary excretion of NAG 24h after a single ferric nitrilotriacetate (Fe-NTA) injection.[30] In animal models of chronic kidney disease, Nrf2 activity exhausted over time despite continuous presence of oxidative stress.[31-33] Therefore, we tested the hypothesis that Nrf2 exhaustion as a result of persistent oxidative stress underlies renal injury observed in chronic iron overload conditions.

To this end, we examined the intracellular effects of long-term iron overload exposure in human conditionally immortalized PTECs (ciPTECs) and the role of the Nrf2 pathway herein.

METHODS

Cell culture

ciPTECs (clone T1, kindly provided by dr. M. Wilmer, Radboud university medical center)[34] were cultured using DMEM HAM's F-12 phenol red-free medium (Thermo Fisher Scientific) containing 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 10 ng/ml epithelial growth factor and 40 pg/ml triiodothyronine (all Sigma Aldrich), 10% (v/v) fetal calf serum (Greiner Bio-one), and 1% (v/v) penicillin-streptomycin (Thermo Fisher Scientific). Cells were cultured at 33°C and 5% CO_2, and grown for 24h at 33°C and 5% CO_2 and 7 days at 37°C and 5% CO_2 prior to experiments.

Iron exposure

Cells were exposed to 0-500 µM ferric citrate (FeC, Sigma Aldrich) for 72h (toxicity array) or 48h (all other experiments) with or without 1 µM trigonelline hydrochloride (Sigma Aldrich). We calculated that already 100 µM FeC could saturate transferrin levels in fetal calf serum[35] with iron and, as such, this would result in ciPTEC iron exposure containing both saturated TBI and NTBI. Cell pellets were collected and stored at -80°C until analysis.

Protein isolation and immunoblotting

Cell pellets were lysed using RIPA buffer (0.15 M NaCl, 0.012 M Sodium Deoxycholate, 0.1% NP40, 0.1% SDS, 0.05 M Tris, pH 7.5, freshly supplemented with protease inhibitors (Complete mini, Roche)). Protein concentration was determined using the Pierce BCA assay kit according to the manufacturer's instructions (Thermo Fisher Scientific). Protein samples were separated on SDS-PAGE gels, transferred to nitrocellulose membrane and incubated with primary antibody overnight at 4°C. After 1h incubation at RT with secondary antibody, proteins were visualized on a LAS-3000 scanner for chemiluminescence (Transferrin receptor 1 (TfR1)) or Odyssey fluorescence scanner (all other proteins). Primary antibodies and dilutions are summarized in Supplementary Table 4.1.

Nucleus enrichment

Cells were resuspended in mild lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 270 mM sucrose, 0.1% NP-40, 20 mM Tris-HCl, pH 7.5, freshly supplemented with 1 mM DTT and protease inhibitors) and disrupted using a Douncer homogenizer. After centrifugation, pellets were incubated with RIPA lysis buffer and supernatant was collected as nuclear fraction.

RNA isolation and quantitative PCR

RNA isolation was performed using TRIzol™ (Thermo Fisher Scientific) according to the manufacturer's instructions. A reverse transcription reaction was performed with 1 µg RNA, 4 µl first strand buffer, 1 µl dNTPs (12.5 mM), 2.04 µl random primers, 2 µl DDT, 1 µl M-MLV (all Thermo Fisher Scientific) and 0.5 µl RNAsin (Promega Corporation). The PCR cycle existed of 20 °C for 10 min, 42 °C for 45 min and 95 °C for 10 min. Quantitative PCR was performed on a CFX96 (Bio-rad) using 4 µl cDNA (10 ng/ml), 10 µl SYBR Green Power master mix (Applied Biosystems) and 6 µl primer mix (containing 1 µM forward primer and reverse primer). The PCR protocol was as follows: 7 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C, and 10 min at 95°C, with a measurement at the end of each cycle. Fold change values were calculated relative to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) using the ΔΔCt formula. Primers are summarized in Supplementary Table 4.2.

Toxicity array

The RT^2 Profiler PCR Array Human Stress & Toxicity Pathway Finder (Qiagen), containing 84 different genes and 5 housekeeping genes was used together with RT^2 SYBR Green qPCR Mastermix (Qiagen) according to the manufacturer's instructions.

Iron assessment

Intracellular iron level were determined using the chromogen bathophenanthroline as described.[36] Iron concentrations were calculated by comparison to a standard curve of ferrous sulfate and corrected for protein concentration.

Oxidative stress measurement

Cells were incubated with 10 µM 2', 7'-dichlorodihydrofluorescein di-acetate (CM-H_2DCFDA, Thermo Fisher Scientific) and FeC for 1h at 37°C. Fluorescence was measured using a Victor X Multilabel Plate Reader (Perkin Elmer).

For oxidative stress staining, cells were incubated with 50 µM CellROX™ Green for 30 min at 37°C, fixed with 4% paraformaldehyde, stained with DAPI (4',6-diamidino-2-phenylindole, 300 µM, Thermo Fisher Scientific) and mounted. Images were taken using a Zeiss Apotome FL microscope and AxioVision software.

Statistical analysis

Data were statistically analyzed using GraphPad Prism 5.03 and presented as mean ± SEM. Results were analyzed by One-Way ANOVA with Dunnett's post test or Student's t-test, where appropriate. Differences were considered statistically significant when $p<0.05$.

RESULTS

Long term iron overload results in Nrf2 pathway activation

In ciPTECs, 48h iron overload exposure significantly and concentration-dependently increased intracellular iron levels ($p<0.05$ for 100 µM and 200 µM, $p<0.001$ for 500 µM FeC compared to control) (Figure 4.1a). High intracellular iron levels are known to decrease TfR1 and induce ferritin protein expression as a result of posttranslational genomic iron responsive element – iron responsive protein (IRE-IRP) regulation[37], which was confirmed in our model in a concentration-dependent manner (Figure 4.1b). Although morphological cell death was not observed (data not shown), chronic iron exposure significantly induced ROS production ($p<0.05$ for 200 µM, $p<0.001$ for 500 µM; Figure 4.1c) and *heme oxygenase 1 (HMOX-1)* mRNA expression ($p<0.05$ for 100 µM, $p<0.01$ for 200 µM and 500 µM), which is a marker for cellular oxidative stress[38] (Figure 4.1d).

Next, we applied an explorative gene expression array to identify pathways involved in long-term iron-induced stress in ciPTECs. Indeed, 72h iron exposure predominantly induced genes regulated by Nrf2 (Figure 2), including *NAD(P)H quinone dehydrogenase 1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM)* and *thioredoxin reductase 1 (TXNRD1)*. Induction of these genes was confirmed by additional quantitative PCR, which showed increased mRNA expression of these genes in a concentration-dependent manner upon 48h of iron exposure, which was statistically significant at 200 µM and 500 µM FeC (Figure 4.3a). Chronic iron exposure induced Nrf2 protein levels in enriched nuclear lysates of ciPTECs, without affecting Nrf2 protein levels in total cell lysates (Figure 4.3b), and moderately increased NQO1 protein levels (Figure 4.3c). Together, these data support activation of the Nrf2 pathway by long-term iron exposure.

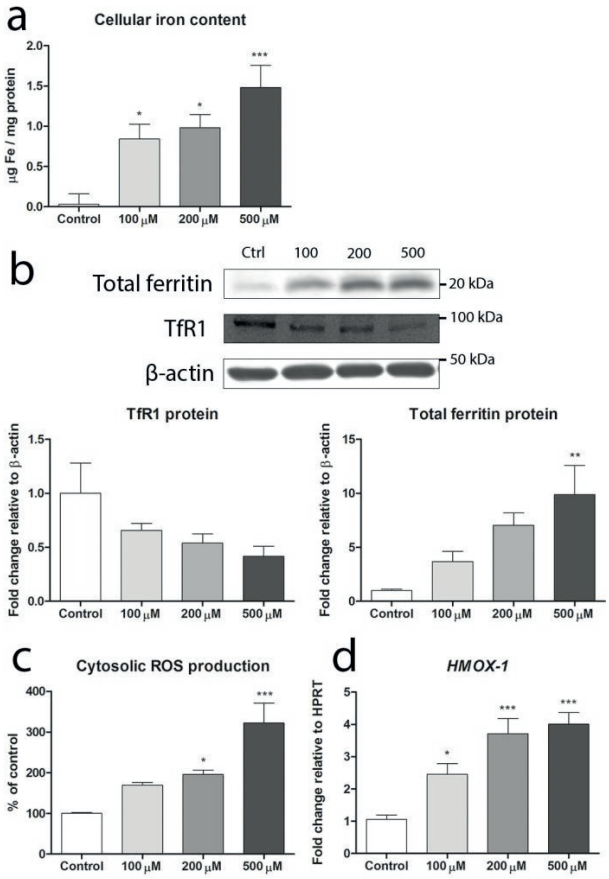

Figure 4.1: Long-term iron uptake and resulting cellular stress in ciPTECs

Intracellular iron concentration (a), total ferritin and transferrin receptor 1 (TfR1) protein levels (b), cytosolic reactive oxygen species (ROS) production (c) and heme oxygenase 1 (*HMOX-1*) mRNA expression after incubation with different concentrations of ferric citrate. Protein content relative to β-actin. Representative images and graphs showing mean of at least three independent experiments. * p<0.05; ** p<0.01; *** p<0.001.

Nrf2 downregulation activates other cellular stress mechanisms

To investigate the effects of iron overload exposure upon Nrf2 exhaustion, we used the Nrf2 inhibitor trigonelline to reduce ciPTEC Nrf2 expression.[39,40] Addition of trigonelline to iron exposure diminished Nrf2 nuclear translocation, as indicated by significantly reduced Nrf2 protein levels in enriched nuclear lysates whereas Nrf2 protein levels in total cell lysates remained unaffected (Figure 4.4a). Moreover, trigonelline also diminished the induction of NQO1 protein levels and decreased mRNA levels of *Kelch like ECH associated protein 1 (KEAP1)*, the Nrf2 binding protein[29] (p<0.05 at 500 µM FeC with trigonelline, compared to 500 µM FeC alone) (Figure 4.4b and c, respectively).

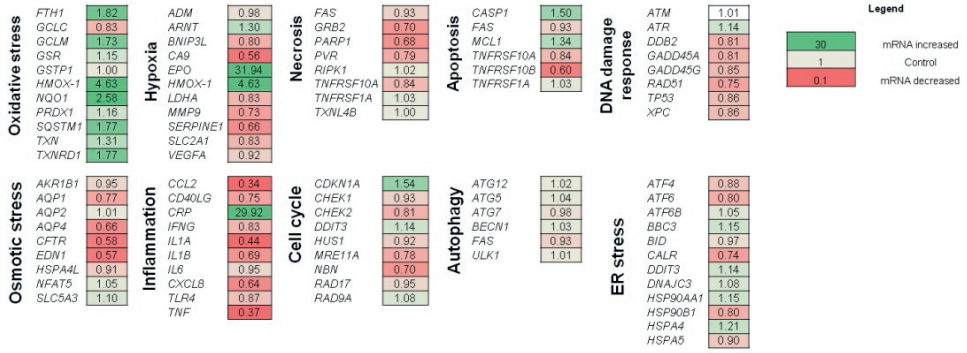

Figure 4.2: Explorative qPCR array results after long-term iron overload exposure in ciPTECs

Results Stress and Toxicity Pathway finder PCR array (Qiagen) after 72h of exposure to 200 µM ferric citrate compared to control. Genes categorized according to manufacturer's protocol. mRNA expression presented relative to housekeeping gene HPRT. Results indicated as increased (green), similar (grey) or decreased compared to control (red). Description of all genes can be found in Supplementary Table 4.3.

Surprisingly, trigonelline co-administration decreased oxidative stress production compared to iron exposure alone (Figure 4.5a), whereas *HMOX-1* mRNA levels were not altered (Figure 4.5b), indicating that cellular stress was still present. To confirm iron uptake by cells co-incubated with trigonelline, protein levels of TfR1 and ferritin were measured. We again observed a concentration-dependent reduction in TfR1 protein levels (Figure 4.5c), similar to previous incubation with iron alone (Figure 4.1b), which suggests a comparable intracellular iron content. However, we observed a striking increase in ferritin protein levels in cell exposed to iron and trigonelline compared to iron alone (Figure 4.5c).

Induction of both ferritin subunits, *H-ferritin* (*FTH)* and *L-ferritin* (*FTL*), was confirmed on mRNA level ($p<0.05$ for 200 µM and 500 µM iron + trigonelline compared to iron alone for *FTH1;* $p<0.01$ for 200 µM and 500 µM iron for *FTL*; Figure 4.5d). To explore potential iron-mediated cell stress mechanisms in Nrf2-downregulated cells, we measured CCAAT-enhancer-binding protein homologous protein (CHOP), an indicator of endoplasmic reticulum (ER) stress. We observed a significant induction of *CHOP* mRNA levels with trigonelline co-administration compared to iron alone ($p<0.05$ for 200 µM iron + trigonelline, $p<0.01$ for 500 µM iron + trigonelline compared to iron alone, Figure 4.5e). This suggests that in case of Nrf2 exhaustion, mechanisms other than oxidative stress may lead to renal injury.

DISCUSSION

Chronically increased luminal iron exposure has been associated with renal tubular injury in patients with systemic iron overload. However, the molecular mechanisms involved in

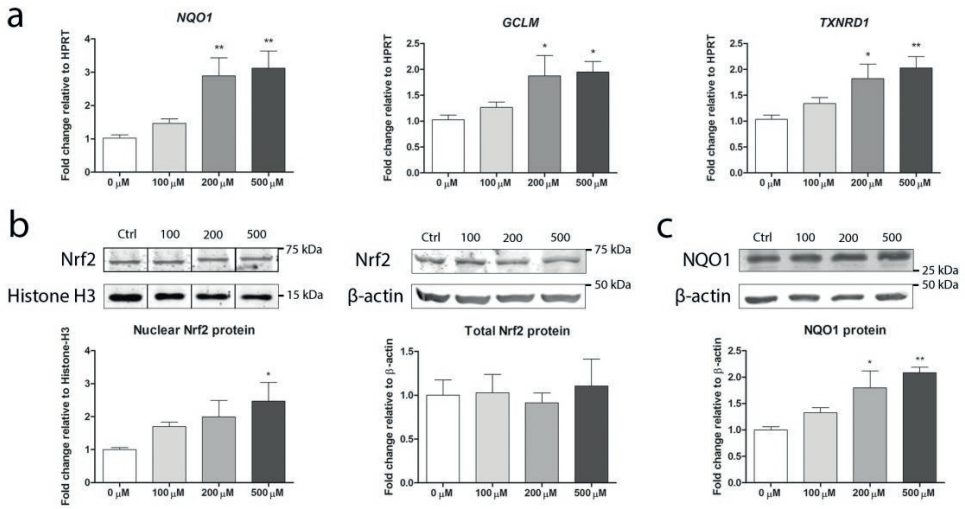

Figure 4.3: Nrf2 pathway activation by long-term iron overload in ciPTECs

NAD(P)H quinone dehydrogenase 1 (NQO1), Thioredoxin reductase 1 (TXNRD1) and *Glutamate-Cysteine Ligase Modifier Subunit (GCLM)* mRNA expression (a), and Nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression in total cell lysate or enriched nuclear fraction, and NQO1 protein expression in total cell lysate (b) after incubation with different concentrations of ferric citrate. Protein content relative to β-actin (total cell lysate) or Histone H3 (nuclear fraction). Representative images and graphs showing mean of at least three independent experiments. * p<0.05; ** p<0.01.

injury during chronic iron overload exposure remained unclear. In this study, we showed that long-term iron exposure resulted in iron loading, oxidative cellular stress and activation of the antioxidative protective Nrf2 pathway in ciPTECs. Moreover, Nrf2 downregulation attenuated ROS production, but simultaneously induced *CHOP* and ferritin. This suggests that other stress-related pathways may be activated during persistent iron exposure once the protective Nrf2 pathway is exhausted (Figure 4.6).

We showed that long-term iron exposure results in PTEC iron loading, which is in agreement with reports of PT iron deposition in systemic iron overload animal models.[41,42] This resulted in IRE-IRP-mediated downregulation of TfR1,[37] suggesting this transporter may not play a major role in PTEC iron uptake. PTECs have other, presumably IRE-IRP-independent, mechanisms that take up iron during increased luminal iron exposure. Potentially, TBI could be taken up by the endocytic megalin:cubilin transporter complex or divalent metal transporters at the plasma membrane mediate NTBI uptake.[6] Long-term iron exposure activated the antioxidative Nrf2 pathway in ciPTECs. The Nrf2 system is known to protect cells from a large variety of electrophilic and oxidative stressors by coordinating the cellular antioxidant response.[43] Indeed, Nrf2 knockout mice are more susceptible to acute Fe-NTA nephrotoxicity than control mice, as shown by enhanced renal tubular necrosis and depletion of glutathione levels in the kidney after Fe-NTA injection.[44] We now show *in vitro* that also chronic iron overload activates the

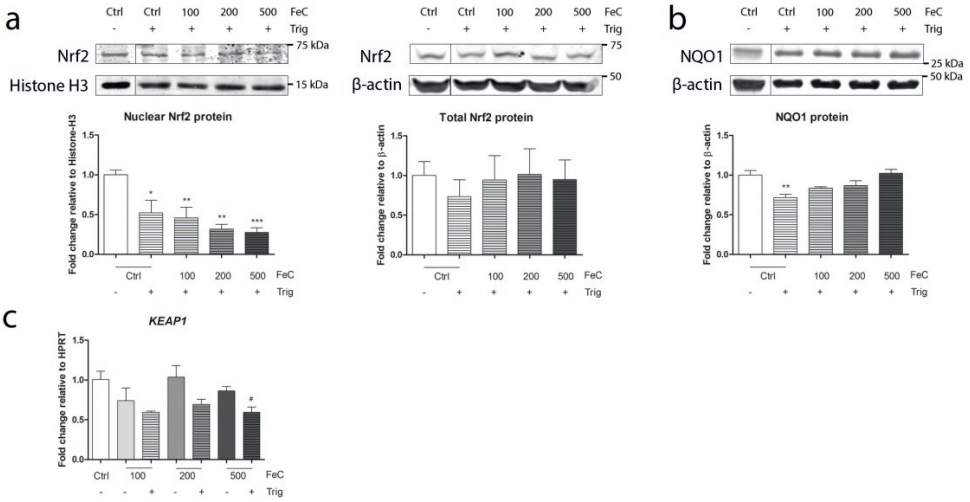

Figure 4.4: Nrf2 downregulation using trigonelline in ciPTECs

Nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression in total cell lysate (a) or enriched nuclear fraction (b), NAD(P)H quinone dehydrogenase 1 (NQO1) protein expression in total cell lysate (c) and *Kelch like ECH associated protein 1 (KEAP1)* mRNA expression after incubation with different concentrations of ferric citrate with (striped bars) or without (open bars) the Nrf2 inhibitor trigonelline (Trig). Protein content relative to β-actin (total cell lysate) or Histone H3 (nuclear fraction). Representative images and graphs showing mean of at least three independent experiments. Iron + trigonelline treated cells compared to control, * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Iron + trigonelline treated cells compared to iron only treated cells, $^{\#}$ $p<0.05$.

antioxidative Nrf2 pathway. Although we observed increased ROS and cellular stress in ciPTECs after long-term iron exposure, these cells did not show signs of cell death, possibly as a result of Nrf2 activation. Previous studies showed Nrf2 exhaustion in murine models of chronic kidney disease.[31-33] Rats subjected to 5/6 nephrectomy developed oxidative stress and lipid peroxidation over time, whereas Nrf2 protein levels in the remnant kidney tissue were decreased rather than increased compared to control animals.[32] Also in Imai rats, a model of spontaneous focal glomerulosclerosis, persistent oxidative stress and impaired Nrf2 activation were observed.[33] During systemic iron overload, failure of Nrf2 activation could potentiate cytotoxicity of persistent iron overload exposure.

Interestingly, we observed decreased ROS levels, induction of ferritin protein levels and enhanced *CHOP* mRNA levels with Nrf2 downregulation by trigonelline and long-term iron exposure. The iron storage molecule ferritin is mainly regulated by iron availability via IRE-IRP regulation and is increased with intracellular iron loading.[37] Increased iron storage in ferritin could prevent intracellular iron from being redox active and could explain our findings of decreased ROS formation.[45] In contrast, Nrf2 KO mice showed increased oxidative stress 6h after Fe-NTA injection.[30,44] This discrepancy could be caused by the iron exposure time, as long-term iron exposure of 48h in our model could

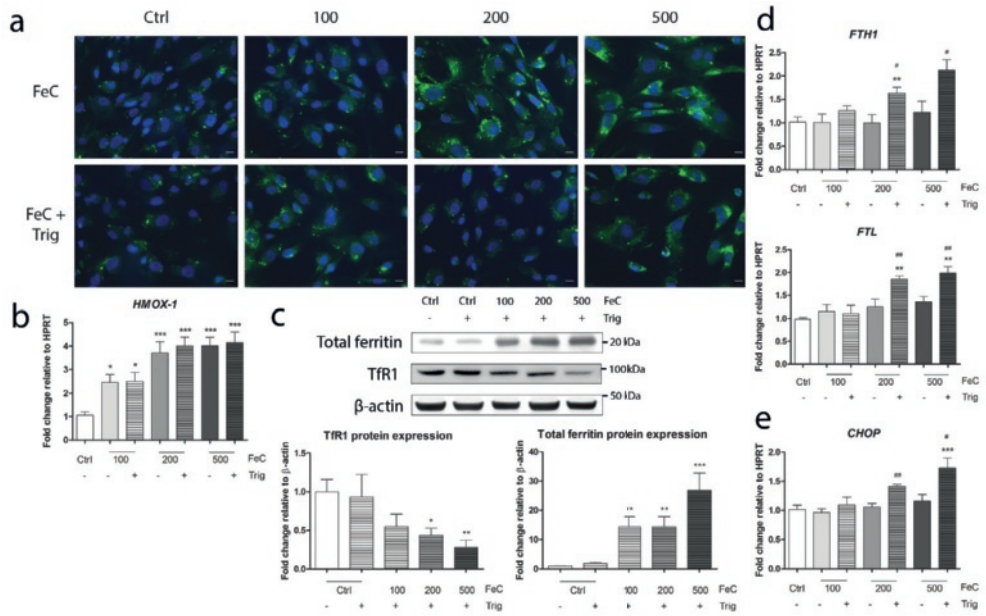

Figure 4.5: Nrf2 downregulation shifts cellular stress pathways

Oxidative stress production (a), *heme oxygenase 1 (HMOX-1)* mRNA expression (b), total ferritin and transferrin receptor 1 (TfR1) protein expression (c), *H-ferritin (FTH1)* and *L-ferritin (FTL)* (d) and *CCAAT-enhancer-binding protein homologous protein (CHOP)* mRNA expression after incubation with different concentrations of ferric citrate with (striped bars) or without (open bars) the Nrf2 inhibitor trigonelline (Trig). Protein content relative to β-actin. Representative images and graphs showing mean of at least three independent experiments. Iron + trigonneline treated cells compared to control, * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Iron + trigonelline treated cells compared to iron only treated cells, $^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$.

model could initiate chronic survival mechanisms that differ from acute cellular stress signalling after iron exposure for only 6h. Potentially, ferritin is not yet increased after 6h of iron incubation, whereas it is after 48h, which could explain differential signalling after acute or chronic iron exposure. Ferritin is also induced by inflammatory cytokines and Nuclear Factor kappa-light-chain-enhancer of activated B cell (NF-κB) signalling, the latter being repressed by Nrf2.[29,46] Therefore, ferritin induction during Nrf2 exhaustion might involve multiple mechanisms, that require further studies. During Nrf2 inhibition, we observed a shift from oxidative stress to potential ER stress signalling as confirmed by induction of *CHOP* mRNA expression. During long-term ER stress, the physiological unfolded protein response (UPR) becomes overruled by apoptotic UPR signalling, which includes the activation of CHOP and other apoptotic UPR genes.[47] In hepatocytes, ER stress activation was reported to induce *H-ferritin* mRNA expression.[48] This suggests that ER stress could also be related to the ferritin induction in ciPTECs during Nrf2 exhaustion. Although ferritin is known to initially safely store iron inside its core, long-term iron

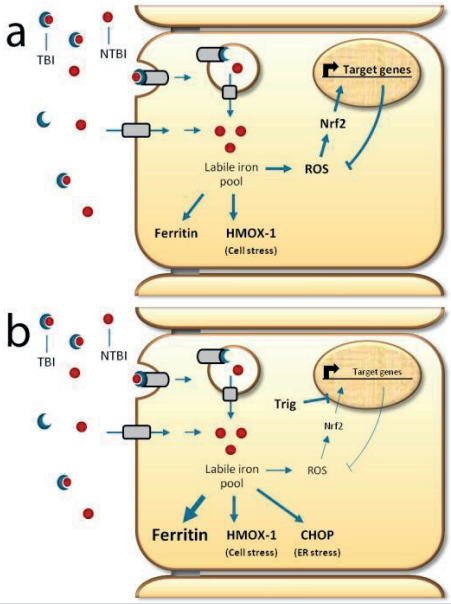

Figure 4.6: Schematic overview of stress pathways during long-term iron overload exposure in proximal tubular epithelial cells

In systemic iron overload, renal tubular epithelial cells are exposed to increased levels of transferrin-bound iron (TBI) and non-transferrin-bound iron (NTBI). Cellular uptake into the labile iron pool results in induction of intracellular iron storage protein ferritin and heme oxygenase 1 (HMOX-1), a marker for cellular stress. Moreover, reactive oxygen species (ROS) are produced, resulting in an anti-oxidative response mediated by Nuclear factor erythroid 2-related factor 2 (Nrf2), balancing oxidative and anti-oxidative conditions (a). When Nrf2 is downregulated by using trigonelline (Trig), ROS production is decreased, whereas HMOX-1 is not affected. Simultaneously, ferritin and CHOP, a marker for endoplasmic reticulum (ER) stress, are induced. This suggests that other stress pathways can be activated during Nrf2 exhaustion, which could potentially mediate chronic iron-induced renal injury (b).

accumulation in ferritin shells may be pro-oxidative.[45] ER stress in tubular epithelial cells is suggested to be involved in the development or progression of chronic kidney disease.[47] Cells subjected to chronic ER stress are cleared via apoptosis, which is frequently seen in chronic kidney disease.[49] Our explorative qPCR array showed that iron overload exposure in ciPTECs induced pro-apoptotic *caspase 1 (CASP1*) but decreased anti-apoptotic *tumor necrosis factor receptor superfamily member 10b (TNFRSF10B*) mRNA expression, suggesting long-term iron exposure may initiate apoptotic cellular signalling. As such, long-term iron overload exposure in patients with systemic iron overload could induce renal PTEC injury through induced ER stress-mediated apoptosis upon intracellular iron accumulation.

In systemic iron overload, PTECs are likely to be a target of iron-mediated injury. Filtered proteins are predominantly reabsorbed by PTECs,[6,50] suggesting these cells are exposed to high iron levels in systemic iron overload. Moreover, PTECs are sensitive to injury due to their high level of energy consumption and mitochondrial content.[51] Nevertheless, in severe iron overload exposure, PTEC injury hampers PTEC iron reabsorption, and increased iron levels pass down the nephron and reach distal tubules. As this could result in iron accumulation and subsequent injury in these epithelial cells, examination of the mechanisms of iron-induced injury in distal tubular epithelial cells is a relevant aspect for future studies.

In conclusion, our findings show that long-term iron exposure activates the Nrf2 pathway in PTECs. Nrf2 exhaustion may result in activation of ER stress and could attribute to chronic iron overload-induced PTEC injury, which may differ from known mechanisms in acute iron-induced kidney injury. Further studies in chronic iron overload models

examining the mechanisms of iron-mediated renal injury could be used to identify treatment strategies to decrease iron-mediated renal injury in patients with systemic iron overload.

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SUPPLEMENTARY INFORMATION

Supplementary Table 4.1: Overview used primary antibodies

Protein	Primary antibody
TfR1	Thermo Scientific 136800, 1:500
Total ferritin	Sigma Aldrich F5012, 1:250
Nrf2	Abcam ab31163, 1:1000
NQO1	Abcam ab34173, 1:1000
Histone H3	Abcam ab24834, 1:1000
β-actin	Sigma A5441, 1:100,000

NQO1, NAD(P)H quinone dehydrogenase 1; Nrf2, nuclear factor erythroid 2-related factor 2; TfR1, Transferrin receptor 1.

Supplementary Table 4.2: Overview used quantitative PCR primers

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
HMOX-1	AGACACCCTAATGTGGCAGC	CTGAGCCAGGAACAGAGTGG
NQO1	TGAAGAAGAAAGGATGGGAGGT	GGCCTTCTTTATAAGCCAGAACA
TXNRD1	TGTCATGTGAGGACGGTCGG	TTCACAAACACAACGGGCAG
GCLM	ACTGACTTAGGAGCATAACTTACC	AAGAATATCTGCCTCAATGACACC
KEAP1	CATCCACCCTAAGGTCATGGA	GACAGGTTGAAGAACTCCTCC
FTH1	GAAGCTGCAGAACCAACGA	CACACTCCATTGCATTCAGC
FTL	CACCTGACCAACCTCCACAG	CGTGCTTGAGAGTGAGCCTT
CHOP	CTTCTCTGGCTTGGCTGACT	TCCCTTGGTCTTCCTCCTCT
HPRT	GCTATAAATTCTTTGCTGACCTGCTG	AATTACTTTTATGTCCCCTGTTGACTGG

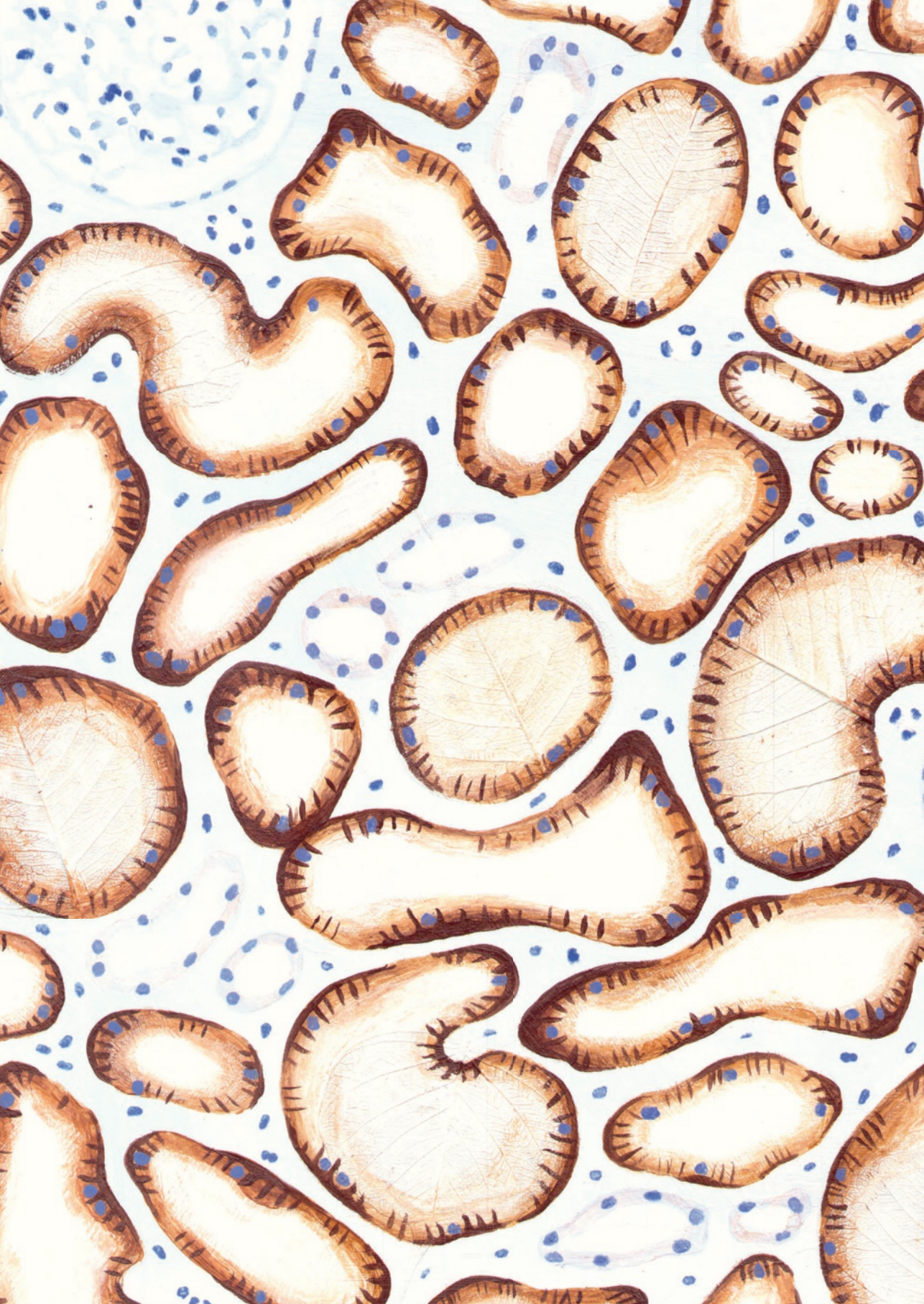
CHOP, CCAAT-enhancer-binding protein homologous protein; FTH1, Ferritin heavy chain; FTL, Ferritin light chain; GCLM, Glutamate-cysteine ligase modifier subunit; HMOX-1, Heme oxygenase 1; HPRT, Hypoxanthine-guanine phosphoribosyltransferase; KEAP1, Kelch like ECH associated protein 1; NQO1, NAD(P)H quinone dehydrogenase 1; TXNRD1, Thioredoxin reductase 1.

Supplementary Table 4.3: Overview target genes in gene expression array

Gene	Description
ADM	Adrenomedullin
AKR1B1	Aldose reductase
AQP1	Aquaporin 1
AQP2	Aquaporin 2
AQP4	Aquaporin 4
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATF4	Activating transcription factor 4 (tax-responsive enhancer element B67
ATF6	Activating transcription factor 6
ATF6B	Activating transcription factor 6 beta
ATG12	Autophagy protein 12
ATG5	Autophagy protein 5
ATG7	Autophagy protein 7
ATM	Ataxia telangiectasia mutated kinase
ATR	Ataxia telangiectasia and Rad3 related kinase
BBC3	BCL2 binding component 3
BECN1	Beclin 1

BID	BH3 interacting domain death agonist
BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
CA9	Carbonic anhydrase IX
CALR	Calreticulin
CASP1	Caspase 1
CCL2	Chemokine (C-C motif) ligand 2
CD40LG	CD40 ligand
CDKN1A	P21
CFTR	Cystic fibrosis transmembrane conductance regulator
CHEK1	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
CRP	C-reactive protein
DDB2	DNA damage-binding protein 2
DDIT3	DNA damage-inducible transcript 3
DNAJC3	DnaJ homolog, subfamily C, member 3
EDN1	Endothelin 1
EPO	Erythropoietin
FAS	Fas
FTH1	H-Ferritin
GADD45A	Growth arrest and DNA-damage-inducible alpha
GADD45G	Growth arrest and DNA-damage-inducible gamma
GCLC	Glutamate-cysteine ligase, catalytic subunit
GCLM	Glutamate-cysteine ligase, modifier subunit
GRB2	Growth factor receptor-bound protein 2
GSR	Glutathione reductase
GSTP1	Glutathione S-transferase pi 1
HMOX1	Heme oxygenase-1
HSP90AA1	Heat shock protein 90kDa alpha, class A member 1
HSP90B1	Heat shock protein 90kDa beta, member 1
HSPA4	Heat shock 70kDa protein 4
HSPA4L	Heat shock 70kDa protein 4-like
HSPA5	Heat shock 70kDa protein 5
HUS1	HUS1 checkpoint clamp component
IFNG	Interferon gamma
IL1A	Interleukin 1 alpha
IL1B	Interleukin 1 beta
IL6	Interleukin 6
IL8	Interleukin 8
LDHA	Lactate dehydrogenase A
MCL1	Induced myeloid leukemia cell differentiation protein
MMP9	Matrix metallopeptidase 9
MRE11A	Double-strand break repait protein
NBN	Nibrin
NFAT5	Nuclear factor of activated T-cells 5
NQO1	NAD(P)H quinone dehydrogenase 1
PARP1	Poly (ADP-ribose) polymerase 1
PRDX1	Peroxiredoxin 1

PVR	Poliovirus receptor
RAD17	Cell cycle checkpoint protein RAD17
RAD51	DNA repear protein RAD51
RAD9A	RAD9 Checkpoint Clamp Component A
RIPK1	Receptor-interacting serine-threonine kinase 1
SERPINE1	Serpin E1
SLC2A1	Glucose transporter 1
SLC5A3	Sodium/myo-inositol cotransporter
SQSTM1	Sequestosome 1
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a
TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TP53	Tumor protein p53
TXN	Thioredoxin
TXNL4B	Thioredoxin-like 4B
TXNRD1	Thioredoxin reductase 1
ULK1	Serin/threonine-protein kinase ULK1
VEGFA	Vascular endothelial growth factor A
XPC	DNA repair protein complementating XP-C cells



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Tubular iron deposition and iron handling proteins in human healthy kidney and chronic kidney disease

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ABSTRACT

Iron is suggested to play a detrimental role in the progression of chronic kidney disease (CKD). The kidney recycles iron back into the circulation. However, the localization of proteins relevant for physiological tubular iron handling and their potential role in CKD remain unclear. We examined associations between iron deposition, expression of iron handling proteins and tubular injury in kidney biopsies from CKD patients and healthy controls using immunohistochemistry. Iron was deposited in proximal (PT) and distal tubules (DT) in 33% of CKD biopsies, predominantly in pathologies with glomerular dysfunction, but absent in controls. In healthy kidney, PT contained proteins required for iron recycling including putative iron importers ZIP8, ZIP14, DMT1, iron storage proteins L- and H-ferritin and iron exporter ferroportin, while DT only contained ZIP8, ZIP14, and DMT1. In CKD, iron deposition associated with increased intensity of iron importers (ZIP14, ZIP8), storage proteins (L-, H-ferritin), and/or decreased ferroportin abundance. This demonstrates that tubular iron accumulation may result from increased iron uptake and/or inadequate iron export. Iron deposition associated with oxidative injury as indicated by heme oxygenase-1 abundance. In conclusion, iron deposition is relatively common in CKD, and may result from altered molecular iron handling and may contribute to renal injury.

INTRODUCTION

Chronic kidney disease (CKD) affects 13% of the population worldwide.[1] Current treatment for CKD patients is mainly aimed at ameliorating renal symptoms, including proteinuria,[2] a major risk factor for disease progression.[3] However, in many patients, this does not prevent progression to end-stage renal disease.[4] The absence of targeted treatment modalities can, at least partly, be attributed to the lack of detailed molecular knowledge on the pathophysiological mechanisms of CKD.

Preclinical studies have suggested a detrimental role for (reactive) iron in the progression of CKD.[5] Increased exposure of renal tubular epithelial cells to iron leads to cellular damage,[6-9] since iron catalyzes highly reactive radical formation in the Fenton reaction.[10] In addition, in patients with various forms of CKD, increased urinary iron levels and renal iron deposition were found,[8,11-19] supporting an association between iron deposition and renal tubular injury. However, it has not been elucidated in which tubular segment iron is deposited.

Human-based studies examining renal iron handling are scarce.[5] It has been suggested that human renal tubular epithelial cells are able to handle iron in physiological conditions, but the localization of proteins involved in cellular iron handling is debated.[20] Transferrin-bound iron (TBI) in the systemic circulation is suggested to be filtered by the glomerulus into the tubular lumen[21,22] and is subsequently completely reabsorbed by endocytic transport.[20] This can be facilitated by transferrin receptor 1 (TfR1) and the megalin-cubilin receptor complex in proximal tubular epithelial cells (PT), and the NGAL receptor (NGALR) in distal tubular epithelial cells (DT).[22-25] Based on *in vitro* and *in vivo* studies, iron transport into the cytosol is reported to involve the putative divalent metal transporters ZIP8 (SLC39A8), ZIP14 (SLC39A14) or divalent metal transporter 1 (DMT1, SLC11A2).[26-28] Subsequently, iron is oxidized by the ferroxidase H-ferritin and stored in L-ferritin, utilized by iron requiring processes, or exported into the blood stream by iron exporter ferroportin (SLC40A1).[20]

Proteinuria as a result of glomerular damage in CKD is linked to tubulointerstitial injury,[29] which is associated with increased filtration of TBI.[5,9,30] Increased exposure of TBI can lead to tubular accumulation of reactive iron as a result of inadequate or disturbed iron handling. In diabetic nephropathy, the kidney can also be exposed to non-transferrin-bound iron (NTBI) derived from filtered TBI as a result of acidification of the filtrate as it passes along the nephron,[5,31] or directly filtered from the circulation.[32] NTBI uptake from the tubular lumen is thought to be mediated by ZIP8, ZIP14 and/or DMT1.[20]

For the first time in human kidney, we characterized associations between the presence and localization of iron deposition, proteins involved in cellular iron handling and tubular injury in kidney biopsies of patients with CKD and healthy controls.

METHODS

Design

Biopsies from patients with CKD and controls were used to examine the presence and localization of iron deposition. Subsequently, a selection of prevalent kidney diseases[33] and controls was made to examine proteins involved in cellular iron handling and tubular injury.

Patients

Renal biopsy material was collected from patients with CKD and potential kidney donors (healthy controls). Biopsies were classified based on clinical presentation of the patient and histological assessment by a trained renal pathologist. Biopsies were collected in University Medical Centre Groningen (the Netherlands (NL)) and Royal Free Hospital (RFH; London, United Kingdom (UK)), during the period of 1996-2014 and 2011-2016, respectively. Biopsies from both hospitals were used to assess iron deposition. For assessment of iron handling proteins and tubular injury, CKD biopsies and healthy kidneys from RFH only were selected, based on tissue availability aiming at age- and gender-matching. Biopsies with excessive tissue damage (>70% of tissue) were excluded.

Procedures and use of anonymized material left over from diagnostic care were performed according to Dutch ethical guidelines. Accordingly, this study did not require approval from an ethical committee and material from patients from UMCG could be used without informed consent. All patients from RFH signed informed consent. All material was collected according to the Declaration of Helsinki.

Histological stainings

Biopsy tissue was embedded in paraffin and cut into 5 µm sections. Renal histology was assessed by Periodic acid-Schiff (PAS) staining and iron deposition by Perls' staining, according to routine staining protocols.

Immunohistochemistry

Stainings were performed using the Bond Polymer Refine Detection system (Leica Biosystems, Newcastle-Upon-Tyne, UK) and provided materials on a Leica Bond Max and Autostainer XL apparatus for DMT1 (Novus Biologicals, Abingdon, UK, H00004891-M01, 1:2000), ZIP8 (Protein Tech, Manchester, UK, 20459-1-AP, 1:500), ZIP14 (Atlas Antibodies, Bromma, Sweden, HPA016508, 1:1000), L-ferritin (Abcam, Cambridge, UK, ab69090, 1:2000), H-ferritin (Abcam ab65080, 1:4000), ferroportin (Abcam ab85370, 1:300) and HO-

1 (Abcam ab13243, 1:100). After antigen retrieval (Bond ER1 or ER2) , sections were incubated with primary antibody for 15 min diluted in Bond Primary Antibody diluent or Antibody diluent with Background Reducing Components (Dako Agilent, Stockport, UK) and secondary antibody for 8 min. Signal visualization was performed with Polymer Refine for 8 min, DAB for 10 min and DAB enhancer (Leica Biosystems). Afterwards, nuclei were counterstained with haematoxylin and images were taken with a Leica DM 2000 microscope connected to a Leica Microsystem Ltd camera, NanoZoomer whole slide imager (Hamamatsu Photonics, Welwyn Garden City, UK) or a VisionTek digital microscope (Sakura Finetek, Alphen aan den Rijn, NL). Appropriate negative control stainings were included for all primary and secondary antibodies (Supplementary Figure 5.1 and 5.2, respectively).

Image analysis

Stainings were evaluated by an expert in renal pathology. Staining intensity was assessed in 5 images per biopsy using the H DAB plugin of ImageJ after correction for background staining. Tubular injury was scored as percentage of the kidney section.

Statistical analysis

Data were analyzed by one-way ANOVA with Dunnett's post test using GraphPad Prism, to evaluate staining intensity and tubular injury in CKD compared to control. Differences were considered statistically significant when $p<0.05$.

RESULTS

Iron deposition in CKD

We found iron deposition in 33% of biopsies from various forms of CKD (n=126), but not in controls (n=8; Figure 5.1, Table 5.1). Iron was deposited in a granular pattern in tubular epithelial cells and in the majority of CKD in both PT and DT. In minimal change disease, iron deposition was found in PT only. We detected iron deposition in kidney disorders characterized by nephrotic glomerulopathy (membranous glomerulopathy, focal segmental glomerular sclerosis (FSGS), minimal change disease), glomerulonephritis (Wegener's disease, anti-glomerular basement membrane (GBM) disease), mesangial glomerular expansion (diabetic nephropathy), and potentially mixed nephrotic and nephritic glomerular injury (lupus nephritis (LN), IgA nephropathy (IgAN), hypertensive glomerulopathy) (Figure 5.1b-k). These findings suggest that renal tubular iron deposition

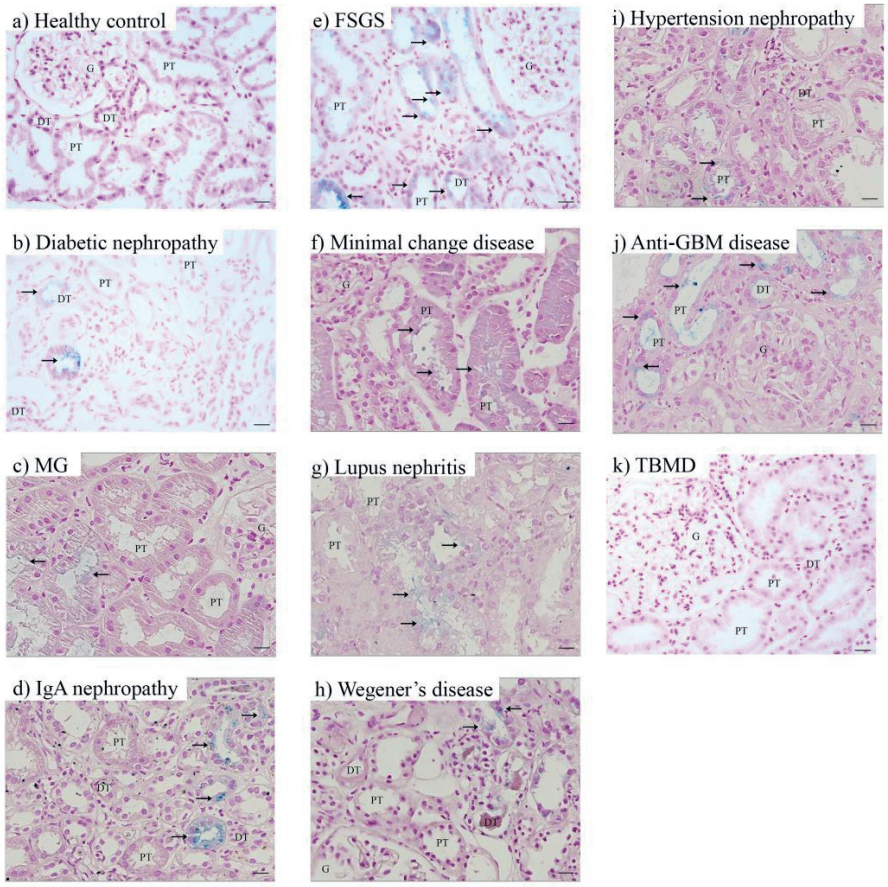

Figure 5.1: Iron deposition in chronic kidney disease

Representative images of Perls' staining in healthy control (a), diabetic nephropathy (b), membranous glomerulopathy (MG; c), IgA nephropathy (d), focal segmental glomerulosclerosis (FSGS; e), minimal change disease (f), lupus nephritis (g), Wegener's disease (h), hypertension nephropathy (i), anti-glomerular basement membrane (GBM) disease (j), and thin basement membrane disease (TBMD; k). Renal structures indicated as glomerulus (G), proximal tubule (PT), distal tubule (DT). Iron indicated with arrows. Scale bar 20 μM.

is a relatively common phenomenon in kidney disorders with glomerular dysfunction of different natures.

Tubular iron handling proteins in healthy kidney

In controls, ZIP8, ZIP14 and DMT1 were detected in both PT and DT (Figure 5.2a-c, Table 5.2). ZIP8 was detected at the apical side of the tubules, whereas ZIP14 was localized intracellularly. DMT1 was observed both apically and intracellularly. Interestingly, L-ferritin, H-ferritin and ferroportin were observed only in PT (Figure 5.2d-f, Table 5.2). Both

Table 5.1: Prevalence of tubular iron deposition in renal biopsies in chronic kidney disease

Kidney disease	Abbreviation	Patients with tubular iron deposition / Total patients (n/n) (%)	Localization of iron deposition
Diabetic nephropathy	DN	6/27 (22)	PT + DT
Membranous glomerulopathy		7/21 (33)	PT + DT
IgA nephropathy	IgAN	7/19 (37)	PT + DT
Focal segmental glomerular sclerosis	FSGS	6/19 (32)	PT + DT
Minimal change disease		3/13 (23)	PT
Lupus nephritis	LN	3/11 (27)	PT + DT
Wegener's disease		5/7 (71)	PT + DT
Hypertension nephropathy		3/4 (75)	PT + DT
Anti-glomerular basement membrane disease		1/1 (100)	PT + DT
Thin basement membrane disease		0/1 (0)	
Total		41/123 (33)	
Healthy control		0/8 (0)	

DT, distal tubule; PT, proximal tubule.

Table 5.2: Tubular localization of iron handling proteins in the healthy kidney

Protein	Proximal tubule	Distal tubule	Tubular localization		
			Apical	Basolateral	Intracellular
ZIP8	+	+	+	-	-
ZIP14	+	+	-	-	+
DMT1	+	+	+	-	+
L-ferritin	+	-	-	-	+
H-ferritin	+	-	-	-	+
Ferroportin	+	-	-	+	+

DMT1, divalent metal transporter 1; +, present; -, not detected.

ferritins showed intracellular orientation while ferroportin was expressed intracellularly and at the basolateral membrane. These findings indicate that PT express proteins for iron import, storage and export, while DT only express proteins involved in iron import in physiological conditions.

Tubular iron handling proteins in CKD

To characterize tubular iron handling during proteinuric CKD, a subset of kidney diseases was selected, including diabetic nephropathy, classified as early and advanced (DNE and DNA, respectively), FSGS, LN and IgAN (Table 5.3). Tubular localization of iron handling proteins in CKD was similar to controls (Figure 5.3-6). Staining intensity, however, differed between CKD and controls. ZIP14 showed comparable intensity in PT and DT, and, therefore, both segments were analyzed together. ZIP14 intensity was significantly increased in FSGS and IgAN compared to control (both $p<0.001$; Figure 5.3g-l, s). In FSGS, ZIP14 intensity was increased in two patients only, while all IgAN biopsies showed

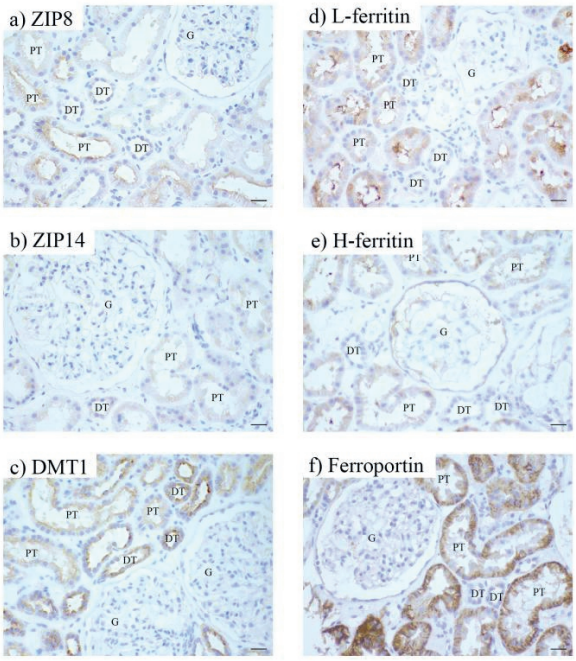

Figure 5.2: Immunohistochemistry of iron handling proteins in healthy kidney
Representatives images of ZIP8 (a), ZIP14 (b), divalent metal transporter 1 (DMT1; c), L-ferritin (d), H-ferritin (e), and ferroportin (f) staining in healthy kidney. Renal structures indicated as glomerulus (g), proximal tubule (PT), distal tubule (DT). Scale bar 20 µM.

Table 5.3: Characteristics of patients included for immunohistochemical staining of cellular iron handling proteins

Kidney disease	Patients (n)	Age (years, mean ± SD)	Gender (M/F)
Control	6	44.8 ± 16.1	3/3
DNE	8	46.6 ± 13.3	4/4
DNA	5	58.4 ± 12.4	4/1
FSGS	5	49.0 ± 18.3	3/2
LN	5	35.2 ± 14.2	1/4
IgAN	5	26.6 ± 9.2	3/2

DNE, established diabetic nephropathy; DNA, advanced diabetic nephropathy; F, female; FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; LN, lupus nephritis; M, male.
Biopsies selected based on tissue availability aiming at age- and gender-matched groups.

enhanced ZIP14 staining. ZIP8 and DMT1 showed differential staining between PT and DT, and were, therefore, analyzed separately. ZIP8 intensity was increased in DT in DNE and FSGS compared to control (both $p<0.01$; Figure 5.3a-f, s), while ZIP8 intensity in PT (Figure

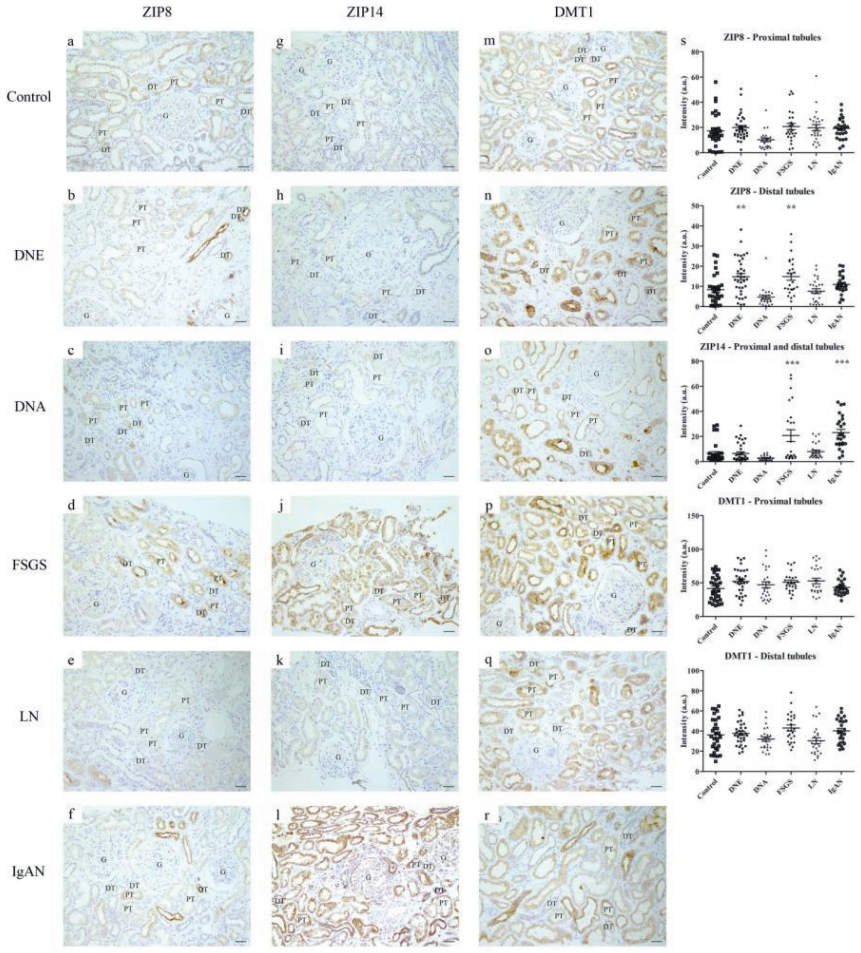

Figure 5.3: Immunohistochemistry of putative iron importers in chronic kidney disease

Representative images of ZIP8 (a-f), ZIP14 (g-l), and divalent metal transporter 1 (DMT1; m-r) staining in control (a, g, m), early diabetic nephropathy (DNE; b, h, n), advanced diabetic nephropathy (DNA; c, i, o), focal segmental glomerulosclerosis (FSGS; d, j, p), lupus nephritis (LN; e, k, q) and IgA nephropathy (IgAN; f, l, r). Intensity in proximal and distal tubules quantified (s). Dots represent all quantified images (5 images per biopsy). Renal structures indicated as glomerulus (G), proximal tubule (PT), distal tubule (DT). Scale bar 40 µM. ** p<0.01; *** p<0.001.

5.3a-f, s) was unchanged. DMT1 intensity in either PT or DT was not different from control (Figure 5.3m-r, s). L-ferritin staining was increased in FSGS (p<0.001; Figure 5.4a, d, m), mainly caused by 2 biopsies, while H-ferritin was overall increased in DNE (p<0.05) and DNA (p<0.001; Figure 5.4g-i, m). Moreover, ferroportin showed decreased intensity in DNE (p<0.01) and DNA (p<0.001) in PT (Figure 5.5). Interestingly, for some CKD biopsies, the observed changes in iron handling protein intensity coincided with iron deposition. In

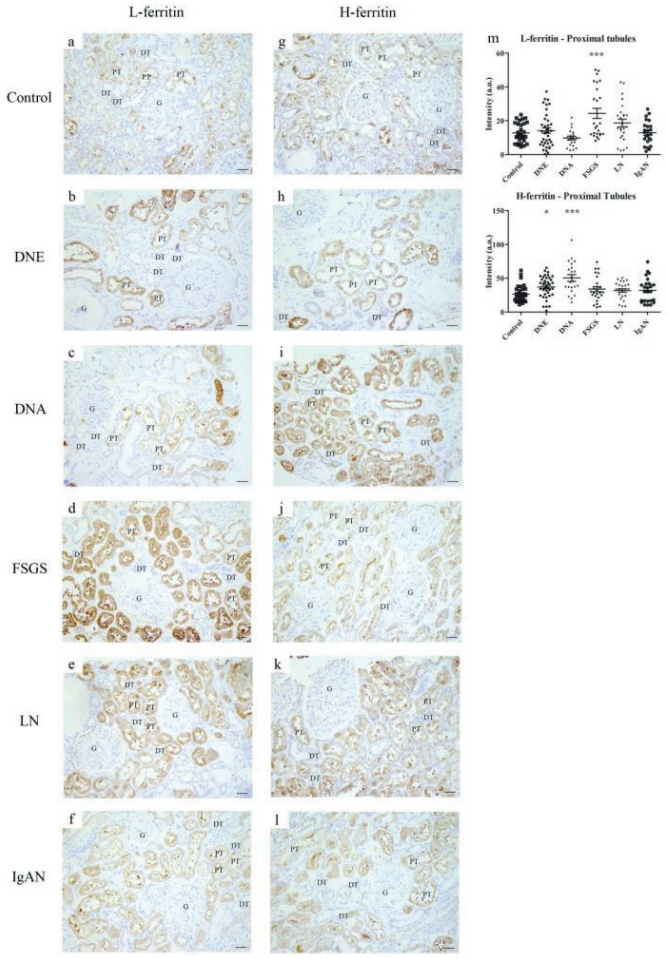

Figure 5.4: Immunohistochemistry of intracellular iron handling proteins in chronic kidney disease
Representative images of L-ferritin (a-f) and H-ferritin (g-l) staining in control (a, g), early diabetic nephropathy (DNE; b, h), advanced diabetic nephropathy (DNA; c, i), focal segmental glomerulosclerosis (FSGS; d, j), lupus nephritis (LN; e, k), and IgA nephropathy (IgAN; f, l). Intensity quantified (m) in proximal tubules. Dots represent all quantified images (5 images per biopsy). Renal structures indicated as glomerulus (G), proximal tubule (PT), distal tubule (DT). Scale bar 40 µM. * $p<0.05$; *** $p<0.001$

FSGS and IgAN biopsies with PT iron deposition, ZIP14 intensity was increased, which was accompanied by increased L-ferritin intensity in FSGS. In DNE and DNA with PT iron deposition, H-ferritin abundance was increased along with decreased ferroportin intensity. In DT, ZIP8 and/or ZIP14 were increased concurrent with iron deposition in DNE,FSGS and IgAN, but neither were observed in DNA. In contrast, no changes in protein abundance of both tubules were seen in any of the LN biopsies despite iron deposition in 2 of the biopsies. These findings are summarized in Figure 5.6.

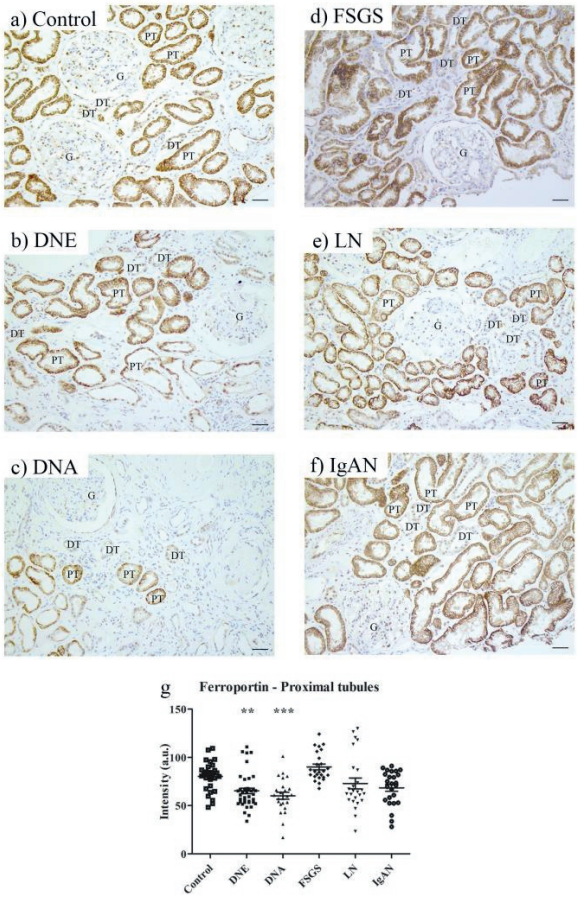

Figure 5.5: Immunohistochemistry of cellular iron export protein in chronic kidney disease
Representative images of ferroportin staining in control (a), early diabetic nephropathy (DNE; b), advanced diabetic nephropathy (DNA; c), focal segmental glomerulosclerosis (FSGS; d), lupus nephritis (LN; e), and IgA nephropathy (IgAN; f). Intensity quantified (g) in proximal tubules. Dots represent all quantified images (5 images per biopsy). Renal structures indicated as glomerulus (G), proximal tubule (PT), distal tubule (DT). Scale bar 40 µM. ** $p<0.01$; *** $p<0.001$.

In conclusion, our findings show associations between tubular iron deposition and abundance of iron handling proteins in most types of CKD, which differ between specific pathologies. Overall, tubular iron deposition was related to increased iron import (ZIP8, ZIP14) in PT and DT, in some CKD biopsies accompanied by increased storage (ferritin) or decreased export (ferroportin) in PT.

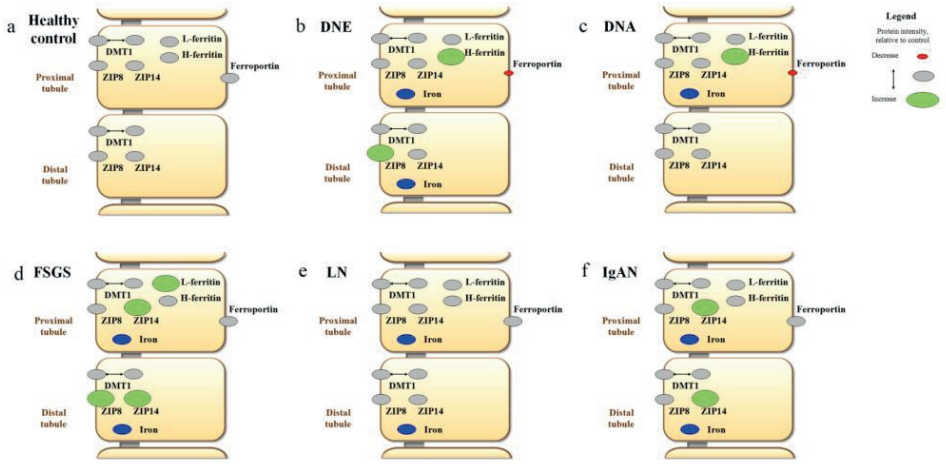

Figure 5.6: Overview of observations on iron deposition and intensity of iron handling proteins in chronic kidney disease

Overview of iron deposition (in blue) and iron handling protein intensity in healthy control (a), early diabetic nephropathy (DNE; b), advanced diabetic nephropathy (DNA; c), focal segmental glomerulosclerosis (FSGS; d), lupus nephritis (LN; e), and IgA nephropathy (IgAN; f). Increased protein intensity compared to healthy controls (in grey) visualized in green, decreased protein intensity in red. *DMT1, divalent metal transporter 1.*

Tubular injury in CKD

Tubular injury was assessed with PAS and heme oxygenase-1 (HO-1) staining. We used PAS staining to assess gross histology and scored renal injury based on tubular atrophy, blebbed tubular structures and irregular tubular cytoplasm (Figure 5.7a-d). Renal damage was increased in DNE and DNA compared to control and moderately elevated in FSGS (Figure 5.7e). HO-1, a marker for oxidative cellular stress,[34] showed comparable intensity in both PT and DT, which was increased in CKD (control < DNE = DNA < IgAN < FSGS = LN; Figure 5.8). Increased HO-1 staining coincided with iron deposition in DNE, FSGS, LN and IgAN, but was also induced in DT of DNA without iron deposition.

In conclusion, we found oxidative cellular stress, but not histological damage, to associate with iron deposition.

DISCUSSION

Increased iron accumulation may be a detrimental factor in progression of CKD, but the mechanisms of iron handling in the human kidney are not fully elucidated. In this study we found iron deposition in one third of biopsies from various CKD disorders, predominantly in pathologies with glomerular dysfunction. We observed that in the healthy kidney, PT

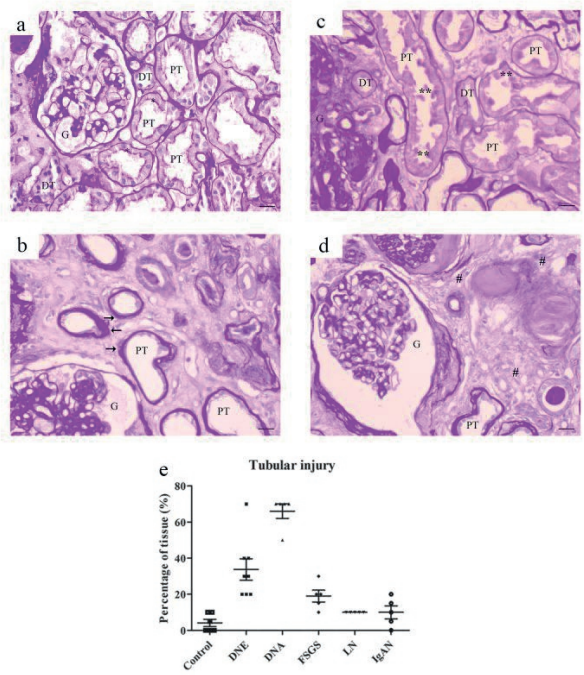

Figure 5.7: PAS staining for tubular injury in chronic kidney disease

Representative images of PAS staining in control (a) or chronic kidney disease showing atrophic tubules with loss of brush border and enlarged basement membrane (b), blebbed tubules (c) and interstitial fibrosis (d). Percentage of tissue with tubular injury scored (E) per patient. Renal structures indicated as glomerulus (G), proximal tubule (PT), and distal tubule (DT). Symbols indicate proximal tubular brush border with asterisk, blebbed tubules with double asterisk, basement membrane with arrow, and fibrosis with hashtag. Scale bar 20 μM.

contain proteins involved in iron import, storage and export, while DT only showed proteins involved in iron import. Associations between iron deposition, intensity of iron handling proteins and tubular injury in CKD were seen, which differed between the pathologies. Nevertheless, in the majority of CKD, tubular iron deposition was accompanied by an increase in iron import proteins ZIP8 and/or ZIP14 in both PT and DT. This coincided with an increase in iron storage proteins or decrease in iron exporter in PT and increased oxidative cellular injury in both PT and DT.

Our data show that iron deposition is a relatively common phenomenon in CKD with glomerular dysfunction. In physiological conditions, TBI filtered by the glomerulus is believed to be almost completely reabsorbed by the tubular epithelium. In this process, PT reabsorb the bulk of filtered proteins and DT play only a minor role.[20] We hypothesize that in nephropathic glomerulopathy or pathologies with mesangial glomerular damage, PT iron reabsorption is increased by large amounts of TBI leaking from the injured

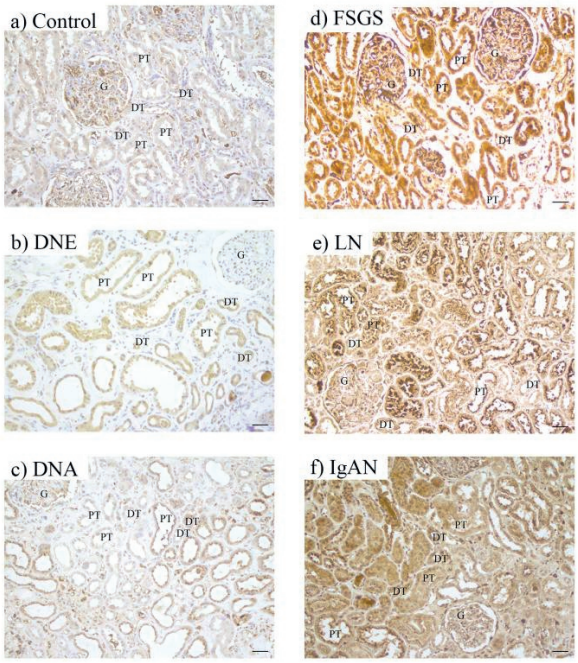

Figure 5.8: Immunohistochemistry of injury marker HO-1 in chronic kidney disease
Representative images of heme oxygenase-1 (HO-1) staining in control (a), early diabetic nephropathy (DNE; b), advanced diabetic nephropathy (DNA; c), focal segmental glomerulosclerosis (FSGS; d), lupus nephritis (LN; e), and IgA nephropathy (IgAN; f). Renal structures indicated as glomerulus (G), proximal tubule (PT), and distal tubule (DT). Scale bar 40 μM.

glomerulus, resulting in local iron deposition. Moreover, when the reabsorption capacity of PT is overwhelmed, DT might also be exposed to increased iron levels, leading to DT iron deposition. In addition, we found iron deposition in pathologies with nephritic glomerular damage, which present with both hematuria and proteinuria.[35] Our results complement previous studies of renal iron deposition in CKD biopsies,[11,12] which was localized to PT lysosomes.[18,19] In our study, we demonstrated iron deposition also in DT in CKD. In addition, we show that iron deposition relates to oxidative cellular injury, as assessed by HO-1 staining. This strengthens the supposition that iron accumulation facilitates highly reactive radical formation that damage membranes, proteins and DNA, and, subsequently, causes tissue injury, which has been reported in renal tubular cells and animal models of CKD,[6-9,36-38] Interestingly, iron reduction via a low-iron diet or treatment with an iron chelator has been reported to reduce renal iron accumulation and tubulointerstitial injury in various CKD animal models.[36-38] Together with findings of increased urinary iron levels and renal iron deposition in patients with CKD,[11-19] this suggests that renal iron loading could contribute to disease progression in patients with

CKD. Biopsies used in this study were taken for diagnostic purposes and are usually at a relatively early stage in disease progression, which is reflected by their tubular injury score. However, renal injury was already more established at the time of biopsy for diabetic nephropathy, especially for DNA. Nevertheless, our findings of iron deposition and associated oxidative cellular injury in FSGS, LN and IgAN biopsies suggest that iron may already be involved in the onset of renal disease and contribute to CKD progression.

We are the first to show ZIP14 and DMT1 in human kidney, whereas ZIP8 was previously described in human kidney.[39] Until now, DMT1 and ZIP14 were only demonstrated in murine PT, and DMT1 and ZIP8 in only mouse and rat DT.[40-46] Luminal TBI reabsorption is suggested to involve TfR1, but TfR1 localization, on the apical or basolateral membrane of tubular epithelial cells, is debated.[31,42,47-49] Since we did not obtain reliable TfR1 stainings, our study is not able to add to the understanding of TfR1 localization. Following entry in the cytosol, iron is known to be oxidized by H-ferritin and stored in L-ferritin. Our results of intracellular expression of both ferritins in PT agree with other studies.[49,50] Cytosolic iron is suggested to be transported back to the systemic circulation via the exporter ferroportin.[20] Although both apical and basolateral localization of ferroportin are shown in murine PT,[31,42,47-49] our human kidney results clearly demonstrate basolateral localization only. This discrepancy might be related to species difference [51]. In our studies, ferroportin was absent in DT, as is shown in rats.[47] The absence of ferritin and ferroportin in DT support that DT only play a minor role in physiological TBI reabsorption. Lack of effective iron storage and export could make these cells vulnerable to iron accumulation and related injury in case of high iron exposure.

For the majority of CKD disorders, we could distinguish an overall increased intensity of ZIP8 or ZIP14 that might have contributed to iron deposition. TfR1 and DMT1 are known to protect cells by limiting iron uptake via iron responsive element-iron responsive protein (IRE-IRP) regulation,[52] but this is not described for ZIP8 and ZIP14.[53] Interestingly, ZIP8 and ZIP14 increase with iron loading in hepatocytes.[28,54] Therefore, ZIP8 and ZIP14 are plausible candidates for unrestricted iron import and subsequent iron loading in kidney tubular epithelium from either endocytosis TBI or direct NTBI transport over the apical membrane.[5,31,32] Future assessment of the intracellular localization and function of ZIP8 and ZIP14 would be valuable to dissect potential iron transport routes in CKD.

We observed decreased ferroportin and concomitant increased H-ferritin intensity in diabetic nephropathy, which may explain PT iron deposition in these patients. Based on IRE-IRP regulation, however, we would expect ferroportin to increase with cellular iron loading.[52] At the systemic level, ferroportin is regulated by hepcidin, which causes ferroportin degradation.[55] Also renal ferroportin protein abundance is shown to decrease with high circulating hepcidin levels.[56] Interestingly, elevated hepcidin levels have been reported in patients with type 2 diabetes in the presence of chronic renal disease, obesity or inflammation,[57,58] which could explain the observed reduction in

ferroportin intensity. Unfortunately, serum hepcidin levels were not available in our study. Moreover, renal biopsies in our study were obtained from patients with either type 1 or type 2 diabetes and the former patients do not have increased hepcidin levels.[59] Our findings of an association between decreased ferroportin and increased H-ferritin are corroborated by studies in human macrophages in which ferroportin silencing led to increased H-ferritin protein levels,[60] suggesting that H-ferritin upregulation could result from decreased ferroportin. Conversely, others showed a reciprocal relationship between H- ferritin and ferroportin by reporting reduced ferroportin mRNA and protein levels in conditional PT H-ferritin knockout mice.[49] Ferroportin is regulated by several multilayered signals, where hepcidin is reported to have a dominant effect.[55] This makes it difficult to determine whether renal iron deposition is due to ferroportin decreases or merely results from other mechanisms. Moreover, because of its multilayered regulation, the contribution of ferroportin to tubular iron loading may be different for various kidney diseases as observed in our study.

Besides ferroportin, also other cellular proteins involved in renal iron handling are regulated by processes beyond iron metabolism, including inflammation and oxidative stress.[55,61,62] These processes are also believed to contribute to the pathophysiology of CKD.[63] Therefore, changes in abundance of proteins we studied cannot solely be attributed to changes in iron handling. Vice versa, iron loading was observed in LN without changes in protein abundance. In these patients, iron accumulation could be mediated by other potential iron transporters that we were not able to include in this study, such as TfR1, the megalin-cubilin receptor complex or NGALR.[22-25] This underlines the complex mechanisms involved in the various CKD pathologies, which may (to various extents) all contribute to changes in renal iron handling proteins.

In summary, our findings in human renal biopsies form the basis for further elucidating renal iron handling in health and disease. Future studies should focus on unraveling the molecular mechanisms of renal iron loading in individual CKD disorders. This will determine whether and how reduction of renal iron accumulation in CKD is a feasible target to halt disease progression.

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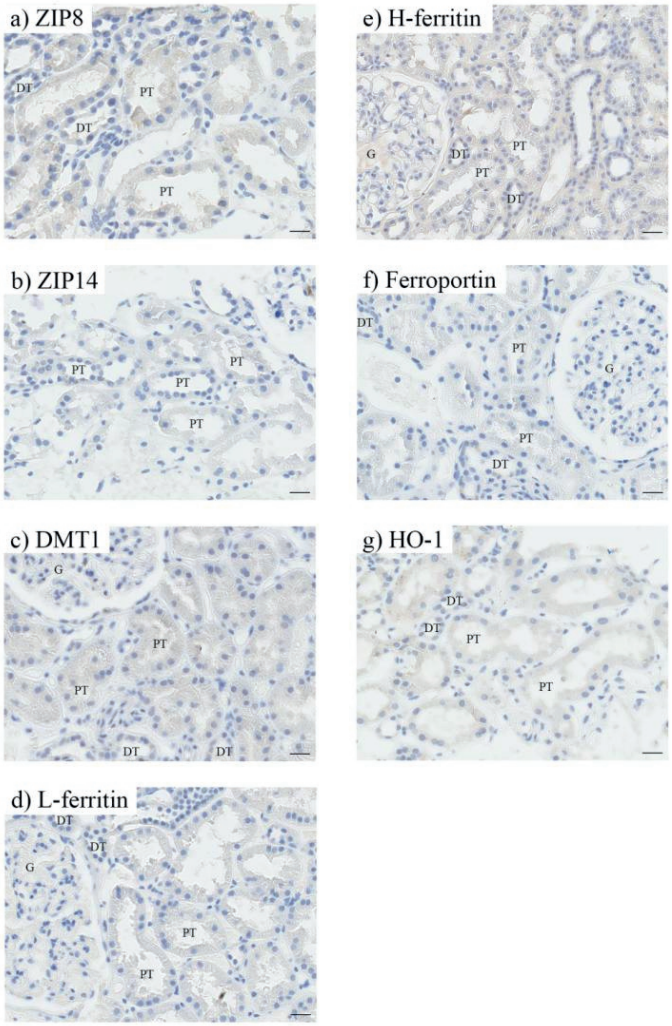
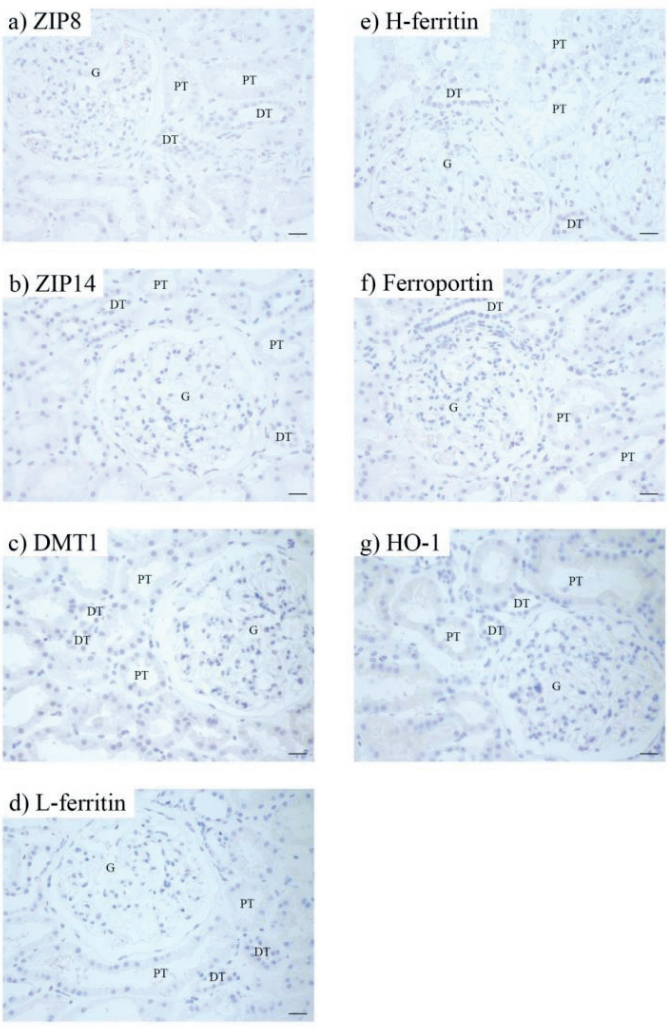
SUPPLEMENTARY INFORMATION



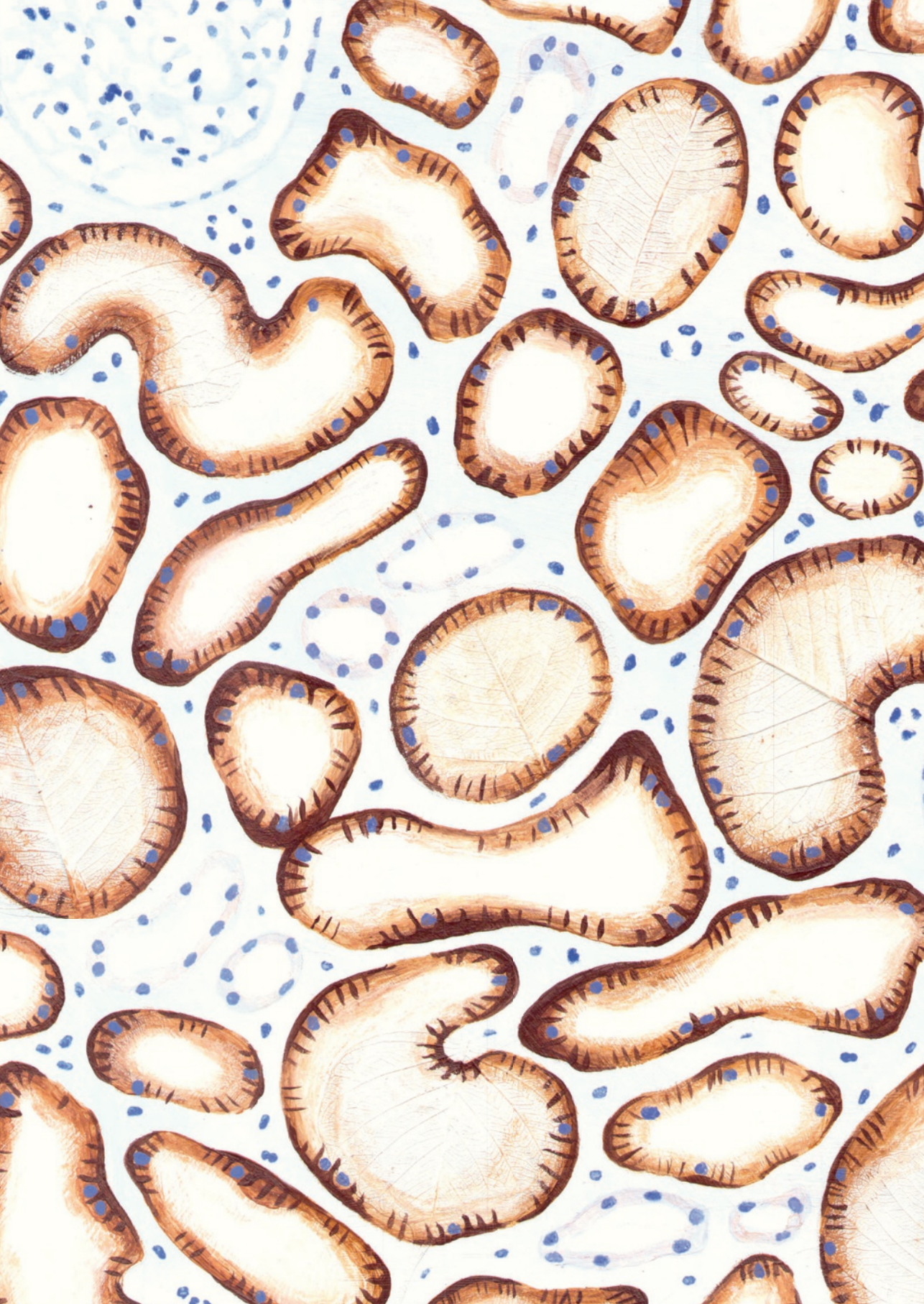
Supplementary Figure 5.1: Primary antibody control stainings

Representative images of primary antibody control (primary antibody replaced by IgG control) for staining procedure of ZIP8 (a), ZIP14 (b), divalent metal transporter 1 (DMT1; c), L-ferritin (d), H-ferritin (e), ferroportin (f) and heme oxygenase-1 (HO-1; g). Renal structures indicated as glomerulus (G), proximal tubule (PT), and distal tubule (DT). Scale bar 20 μM.



Supplementary Figure 5.2: Secondary antibody control stainings

Representative images of secondary antibody control (omission of primary antibody) for staining procedure of ZIP8 (a), ZIP14 (b), divalent metal transporter 1 (DMT1; c), L-ferritin (d), H-ferritin (e), ferroportin (f) and heme oxygenase-1 (HO-1; g). Renal structures indicated as glomerulus (G), proximal tubule (PT), and distal tubule (DT). Scale bar 20 μM.



6

General discussion

INTRODUCTION

Historically, iron handling and transport have mainly been studied in organs including the liver and gut. However, iron handling in the kidney has increasingly gained attention over the past few years due to findings of increased urinary and renal iron levels associated with renal injury.[1] In this thesis, we studied the molecular mechanisms of renal iron handling and its potential toxicity in human health and in patients with systemic iron overload and chronic kidney disease (CKD). We showed that glomerular filtration and tubular reabsorption both can contribute to excretion of iron in human urine and characterized uptake mechanisms for transferrin-bound iron (TBI) and non-transferrin-bound iron (NTBI) in human proximal tubular (PT) epithelial cells (PTECs). Moreover, we demonstrated that long term iron exposure can result in renal cytotoxicity in human PTECs. In addition, we demonstrated tubular iron accumulation and altered renal iron handling protein expression in human CKD. In this chapter, our findings and their implications will be discussed and placed into the perspective of future research.

REDUNDANCY

Renal tubular iron uptake reaches beyond single transporters and is a complex interplay of multiple proteins. Iron transporters transport either TBI or NTBI with different affinities. The abundance and localization, both intracellular and tubular, differs for the various iron transporters. Combined, all transporters ensure that tubular iron is reabsorbed, independent of the iron species and competition of other ligands.[2,3] This underlines the assumption that the kidney is thought to preserve the body's iron levels to ensure that excretion of this scarce metal in urine is limited in physiological conditions.[4] Iron transport redundancy complicates assessment of the role of individual transporters in renal iron handling in health and disease. Redundancy of iron transport may differ between PTECs and distal tubular (DT) epithelial cells (DTECs), and may also be different in pathological conditions. Insights in renal iron transport and redundancy will increase our understanding of renal iron handling and could also be relevant in clinical settings to inhibit renal iron uptake. It has to be determined how strong iron transport redundancy is in disease conditions and whether one transporter potentially dominates, in order to establish if inhibition of iron uptake is feasible as a therapeutic target to prevent renal iron accumulation.

Redundancy in TBI transport

TBI filtered into the tubular lumen can be reabsorbed by endocytic transporters transferrin receptor 1 (TfR1) and the megalin:cubilin:amnionless receptor complex at the apical membrane in proximal tubules (PTs).[5] We confirmed previous findings from rat studies that TBI uptake in PTs predominantly takes place from the apical cellular side and is mediated by TfR1 in human conditionally immortalized PTECs (ciPTECs) (**Chapter 3**).[6-8] Moreover, in ciPTECs, our observations suggest that TfR1 is likely to play a larger role in TBI uptake than the megalin:cubilin:amnionless receptor complex in physiological conditions. Also in mouse renal progenitors, TfR1 was of vital importance for iron acquisition. TfR1 KO renal progenitors explanted at E11 (embryonic day 11) were not able to grow in medium containing holo-transferrin,[9] suggesting TfR1-mediated iron uptake is vital for renal tubular cells. Therefore, we postulate that TfR1 is the main transporter for TBI uptake in physiological conditions. However, in pathological conditions of iron overload, the megalin:cubilin:amnionless receptor complex is likely to play a larger role than TfR1, since in these conditions TfR1 abundance is decreased by iron responsive element – iron responsive protein (IRE-IRP)-mediated regulation.[10]

After TBI endocytosis, iron is released from transferrin and transported into the cytosol. Although divalent metal transporter 1 (DMT1), ZIP8 and ZIP14 are shown to mediate this process in hepatocytes, macrophages and neurons,[11-16] we showed that ZIP14, but not ZIP8, is involved in TBI-derived iron transport towards the cytosol in ciPTECs (**Chapter 3**). ZIP14 silencing did not largely inhibit TBI-derived iron uptake in our cells. Moreover, ZIP14 was found to be dispensable in renal iron loading after TBI exposure in HFE or HFE2 KO mice, models for HFE-related hereditary hemochromatosis (HFE-HH),[17] which suggests that additional transporters are involved in PTEC endocytic iron uptake. DMT1 is a potential candidate for PTEC endosomal iron transport, which we detected in human kidney and localized to ciPTECs endosomes (**Chapter 3**, **Chapter 5**). Four splice variants of DMT1 have been described, based on alternative splicing of both the N- and C-terminus, which all have been detected in mouse and rat kidney,[7,18] but not yet in human kidney. Interestingly, only two DMT1 isoforms contain an IRE element,[18] and differential expression of these isoforms, *i.e.* expression at plasma membrane, endosomes and/or lysosomes, has been described in hepatocytes, epithelial cells and Madin-Darby Canine Kidney (MDCK) cells,[19-21] suggesting that the different DMT1 variants may display distinct iron-handling functions within tubular cells. Using an antibody targeting all four isoforms, DMT1 was detected in late endosomes/lysosomes in rat PTECs where it colocalized with fluorescently labelled transferrin, suggesting DMT1 may be involved in endosomal iron transport in kidney tubular cells.[7] Another potential candidate for endosomal iron transport is mucolipin-1 (transient receptor potential cation channel, mucolipin membrane 1, TRPML1). This ion channel is shown to export calcium or iron from late endosomes and early lysosomes in hepatocytes,[22] but was also detected in the human kidney.[23]

Redundancy in NTBI transport

Redundancy is also shown for NTBI uptake at the plasma membrane, which is filtered into the renal tubular lumen in conditions of systemic iron overload.[5] ZIP8, ZIP14 and DMT1 have all three been described to facilitate NTBI uptake in various non-renal cell types and experimental set ups,[2,12,16,24-27] in addition to which we have now identified ZIP8 and ZIP14 as NTBI uptake transporters in ciPTECs. The redundancy between ZIP14 and ZIP8 in **Chapter 3** is supported by a study with whole body ZIP14 KO mice, in which loss of ZIP14 did not abolish renal iron loading subsequent to NTBI exposure.[17] Redundancy in iron transport by ZIP8 and ZIP14 is not surprising, considering that these transporters show similarities in metal transport characteristics.[28] Both metal transporters transport a large variety of divalent metals, e.g. manganese, cadmium, zinc and iron, but not copper, and are metal/bicarbonate symporters.[2,15] Using iron transport studies in *Xenopus* oocytes, mouse ZIP14 was shown to have a higher affinity for iron transport, *i.e.* $K_{0.5}$ (iron concentration at which transport is half maximal) of 2.2 µM for ZIP14 vs 0.7 µM for ZIP8,[2,15] but apparently the iron transport abilities of ZIP8 were sufficient to compensate for ZIP14 downregulation in our ZIP14 silencing experiments in ciPTECs. Potentially, this could be facilitated by increased ZIP8 abundance, since ZIP8 mRNA expression levels were higher than ZIP14 in mouse kidney.[29] Furthermore, other transporters may also mediate NTBI uptake in tubular epithelial cells. We detected ZIP8, ZIP14 and DMT1 in DTs in human kidney biopsies (**Chapter 5**), which potentially also internalize NTBI. Moreover, DTECs also express Transient receptor potential cation channel subfamily V member 5 (TRPV5, or ECaC) and other calcium channels, which could mediate iron uptake. Transport studies with the TRPV5 isoform present in pufferfish, which is shown to display all structural features of mammalian TRPV5, reported that TRPV5 is also permeable for iron.[30] The TRPV5 protein is detected in rat kidney,[31] indicating that TRPV5 may also serve as a transporter involved in DTEC NTBI acquisition. Alternatively, both L-type and T-type voltage-dependent calcium channels (LTCC and TTCC, respectively) have been reported to facilitate NTBI uptake in cardiomyocytes during iron overload.[32-34] Both LTCCs and TTCCs have been demonstrated on the apical membrane of rat DTECs,[35,36] suggesting these channels may mediate NTBI uptake in the kidney. To our knowledge, no other candidates for NTBI transport have been described in PTs.

Implications of iron transport redundancy

Redundancy of metal transport by ZIP8 and ZIP14 can also be illustrated by findings in patients with rare congenital disorders of manganese metabolism resulting from ZIP8 or ZIP14 deficiency. Mutations in the gene for ZIP8 (SLC39A8) result in intellectual disability, cerebellar atrophy or features of Leigh-like mitochondrial disease.[37] Moreover, these patients suffer from serum manganese deficiency and urinary manganese wasting.[38] However, serum iron levels were within normal range.[39] In contrast, ZIP14 deficiency,

resulting from mutations in the SLC39A14 gene, is recently reported to result in hypermanganesemia and childhood-onset parkinsonism-dystonia,[40] potentially as a result of reduced biliary manganese clearance.[41,42] Interestingly, similar to findings in ZIP8 deficient patients, serum iron indices are normal.[41,42] The findings in these patients suggest that loss of ZIP8 or ZIP14 iron transport function was compensated for and physiological iron metabolism was not affected.

Inhibition of iron uptake transport by renal tubular epithelial cells could potentially be a new therapy to reduce renal tubular iron accumulation. Moreover, this could also be used to reduce systemic iron levels in patients with systemic iron overload, by enhancing excretion of iron in urine (as discussed in **Chapter 2**). However, such an approach is challenging considering iron transport redundancy. Moreover, transporters involved in renal iron handling are not specific for the metal iron. Although the megalin:cubilin:amnionless transporter complex is involved in TBI reabsorption in PTECs,[6] inhibition of this transporter complex may result in severe side effects since this transporter complex also reabsorbs a large variety of other proteins.[43] Similarly, ZIP8 and ZIP14 transport various other divalent metals in addition to iron.[28]

TUBULAR IRON HANDLING

The presence of iron handling proteins has been described in PTs, DTs as well as other tubular segments such as the loop of Henle and collecting duct,[5] but their functioning in human renal iron reabsorption have barely been described. In healthy conditions, PTs reabsorb the bulk of proteins (>80%) from the glomerular filtrate, limiting urinary protein excretion.[5] Since PTs are adjacent to the glomerulus and play such a key role in protein reabsorption, it is suggested that predominantly PTs reabsorb filtered TBI in health. This explains the expression of TfR1 and divalent metal transporters DMT1 and ZIP14 on the luminal tubular side, as well as cellular expression of proteins involved in iron storage and export (**Chapter 3**, **Chapter 5**). The critical role of PT TBI reabsorption was underlined by our results of urinary iron and transferrin excretion in patients with a dysfunction of PT endocytic reabsorption, termed Fanconi syndrome (**Chapter 2**), in line with findings by others.[44-46] We hypothesize that PTs are not able to take up all filtered TBI in case of PT dysfunction and/or injury, and, subsequently, TBI is passed down the nephron towards DTs. The role of PTs in iron reabsorption is illustrated in patients with nephrotic chronic kidney disease (CKD), in whom injury to the glomerulus leads to increased TBI levels in the tubular lumen, resulting in PTEC iron deposition (**Chapter 5**).[47-49] PT iron reabsorption can become overwhelmed or saturated, as shown by increased transferrin and iron excretion in patients with nephrotic CKD.[44,50-55]

Due to the enormous PT reabsorption capacity, DTs seem to play only a minor role in physiological iron reabsorption from the glomerular filtrate.[5] However, it is not completely clear how DTs meet their physiological iron demand, necessary for intracellular processes. It is most plausible that DTECs attain the iron required for cell functioning from the circulating TBI, *i.e.* via basolateral uptake. Rat and human DTECs are reported to express TfR1,[56,57] which was detected at the basolateral membrane in MDCK cells, a cell line believed to originate from a DT nephron segment,[58,59] suggesting that TfR1 mediates TBI uptake in DTECs at the basolateral membrane. Basolateral DTEC iron acquisition could be distinct from a basolateral iron uptake mechanism in PTECs. Luminal iron reabsorption in PTECs is likely to provide sufficient iron levels for normal PTEC physiology, such that an additional basolateral iron acquisition mechanism may be designed for low amounts of iron. Indeed, TfR1 was recently detected at the basal membrane of PTECs and DTECs in human kidney biopsies, but its abundance in DTECs was more intense than in PTECs.[57] In contrast, in pathological conditions when iron travels down the nephron resulting from PT iron reabsorption insufficiency, DTs may be luminally exposed to high iron levels. Our immunohistochemistry stainings in healthy human kidney biopsies (**Chapter 5**) and previous findings of others[60-64] show that DTECs contain iron uptake transporters, such as NGAL receptor (NGALR), DMT1, ZIP8 and ZIP14, allowing TBI to be taken up by these tubular cells. In our biopsy study, we could not detect ferritin orferroportin in DTECs (**Chapter 5**). However, since every cell type in the human body is dependent on iron, iron storage in ferritin is also essential for every cell,[65] and thus, it is very unlikely that DT do not possess ferritin. Indeed, mRNA expression of L- and H-ferritin mRNA was recently reported in a mouse cortical collecting duct cell line ($mCCD_{cl1}$).[66] Likely, the abundance of ferritin in the DTECs is much lower compared to the PTECs, which is in line with the presumed low iron fluxes in DTECs compared to PTECs. The absence of ferroportin in DTECs is supported by findings of others. Ferroportin could not be detected in DTECs of rat biopsies[67] or $mCCD_{cl1}$ cells (R. van Swelm, unpublished observations). Since ferroportin is the only known mammalian iron exporter, DTECs appear to lack an effective mechanism to export iron, which could render the DT vulnerable for iron accumulation and potential associated injury.

Clinical implications of tubular iron handling

We observed iron accumulation in PTECs and DTECs in human CKD (**Chapter 5**), suggesting that these tubules are at risk for iron-mediated renal injury. Also in animal models of CKD, iron deposition was localized to PTECs and DTECs.[68] However, to date it remains unknown whether the onset of renal injury in CKD could be (partly) attributed to iron. The same knowledge gap can be identified in systemic iron overload. Persistent systemic iron overload has been observed to lead to PT and DT iron deposition in patients with HFE-HH or β-thalassemia syndromes,[69-73] but it is not yet clear whether the presence of iron

deposition in these tubular segments also implies that iron plays an active role in renal disease or injury process.

Altogether, we show in this thesis that PTECs and DTECs have differential iron transport mechanisms that may precede iron-mediated injury in these tubular segments. However, these findings also underline that differential iron reabsorption in PTECs and DTECs in pathology is complex and more evidence is needed to elucidate the role of these various transport mechanisms in the onset and/or progression of iron-mediated kidney injury.

IRON CYTOTOXICITY

By exposing ciPTECs to long term iron overload, we showed that iron can induce formation of cytosolic reactive oxygen species (ROS; **Chapter 4**). Oxidative stress is frequently mentioned as a mechanism of iron toxicity, based on the ability of iron to catalyze ROS formation in the Fenton and Haber Weiss reaction.[74] However, oxidative stress is nowadays used as a generic concept encompassing numerous, yet diffuse and nonspecific, cellular processes, whereas this term was initially introduced to specifically indicate cellular redox biology.[75] Cellular redox sources are numerous, ranging from byproducts of electron transport chain complexes up to intentional ROS production as cellular defense mechanisms, and comprise various labile oxygen and nitrogen species that can arise in different cellular organelles.[76] As such, it is very difficult to identify changes in either oxidative stress or cellular redox biology and to pinpoint how exactly iron exposure affects these cellular processes at the biochemical level. Furthermore, the cellular redox balance is determined by production of oxidative species on the one hand and the protective antioxidative defense on the other hand. As soon as the oxidants dominate, these harmful molecules cause injury to cellular structures including DNA, proteins and lipids.[77] The transcription factor Nuclear factor-erythroid 2-related factor 2 (Nrf2) is known to be induced by oxidants and coordinates the protective cellular antioxidative response.[78] In our studies, iron overload in ciPTECs induced Nrf2 nuclear translocation as well as activation of its targets (**Chapter 4**). Nrf2 is reported to protect from short term iron-induced renal injury in PTs,[79,80] suggesting that the antioxidative Nrf2 response may also be involved in protection against renal cellular injury caused by iron in chronic exposure conditions. Oxidative stress is inseparably linked to inflammation, another detrimental process associated with iron exposure,[81] and both are common enhancers of renal injury.[82] The Nrf2 pathway shows crosstalk with Nuclear Factor kappa-light-chain enhancer of activated B cell (NF-κB) signaling, which is the main transcription factor regulating the cellular inflammatory response.[83] Iron is also suggested to directly affect NF-κB signaling.[84] Furthermore, both Nrf2 and NF-κB show cross talk with endoplasmic reticulum (ER) stress

signaling, which is a precursor of apoptotic cellular processes.[85] ER stress can also be induced by ferroptosis, a pathway of regulated cell death which is specifically dependent on iron.[86] Being distinct from apoptosis and necrosis, ferroptosis may result from inappropriate antioxidant mechanisms and consequent accumulation of lipid peroxides.[87] Altogether, this illustrates that iron-mediated renal injury may be a complex interplay of several interacting cellular signaling pathways and processes. As a result, it remains to be determined if there is a dominant mechanism that facilitates the deleterious effects of iron on the cellular level, or if oxidative stress, inflammation, ER stress and ferroptosis collaboratively contribute to this process. Moreover, future studies are required to investigate if the harmful effects of iron are caused by the same mechanisms in the various pathologies characterized by renal iron loading, such as CKD and systemic iron overload. Activation or modulation of related detrimental processes, such as oxidative stress and inflammation, by other causes may affect the magnitude and/or mechanisms of iron toxicity. For example, although we have identified intensified heme oxygenase 1 (HO-1) protein abundance, a marker for cellular oxidative stress, in various CKD pathologies, its expression level varied among these pathologies (**Chapter 5**). As a result, the ability of iron to induce oxidative cellular damage may be different between the pathologies.

Clinical evidence of renal iron toxicity

Although iron has cytotoxic abilities and iron exposure is associated with renal injury in various pathologies,[1] it remains to be determined to what extent iron is involved in the onset or progression of kidney injury. During systemic iron overload, it is plausible that increased iron levels in the kidney provoke tubular injury. The degree of iron loading in the circulation, represented by the degree and duration of blood transfusion in patients with β-thalassemia major, was found to correlate with urinary excretion of N-acetyl-β-D-glucosaminidase (NAG), a marker for renal tubular damage.[88,89] Moreover, iron levels in the human parenchymal tissues, represented by serum ferritin levels, correlated with urinary NAG concentrations and activity.[90,91] Serum ferritin levels were also found to correlate with the degree of renal iron accumulation.[92] Interestingly, it has been suggested that increased serum ferritin levels can be caused by renal tubular iron loading.[93,94] indicating that iron overload inside tubular cells may be involved in causing renal injury during systemic iron overload. Interestingly, the causal role of iron in kidney injury was highlighted by findings that tubular injury markers, such as NAG, were no longer increased when β-thalassemia major patients were treated with iron chelator deferoxamine.[91,95,96] In CKD, iron is likely to play a role in progression of renal injury rather than the onset. The pathogenesis of CKD is multifactorial and complex, often including hypertension, diabetes, obesity or exposure to drugs or toxins.[97] Increased iron levels in the kidney could be considered as a toxin and initiate renal injury, although this is highly unlikely based on the knowledge that systemic iron levels are within the normal range or rather indicative of

iron deficiency at the initial stages of CKD.[98] Therefore, a role of iron in CKD is more likely to be-mediated by increased TBI filtration as a result of glomerular leakage and proteinuria, potentially aggravating tubulointerstitial injury. In experimental studies with human subjects with CKD, chelation therapy with EDTA was shown to reduce a decline in kidney function in patients with chronic renal insufficiency.[99] Moreover, deferiprone treatment was successful in reducing proteinuria in patients with diabetic nephropathy or glomerulonephritis.[100] These studies suggest that iron is involved in the progression of CKD.

The assessment of the clinical relevance of iron in the initiation or progression of kidney injury requires studies of these processes over time for each of the relevant disorders. Prospective, longitudinal, observational human studies should clarify if the extent of renal injury associates with urinary iron concentrations and quantification of iron deposition in the kidney.

TREATMENT MODALITIES

In patients with HFE-HH and β-thalassemia major, current iron removal therapies consist of phlebotomy and iron chelation therapy, respectively, but these therapies are not without burdens and complications.[101,102] Phlebotomy is successful in removing large quantities of circulating iron and inducing a gradual decline in body iron stores,[103] but requires weekly or biweekly visits to the hospital for a long period of time.[101,104] Moreover, iron chelation therapy results in urinary and/or fecal excretion of iron-chelator complexes and reduces systemic iron levels,[105] but adverse effects such as growth restriction, gastrointestinal symptoms and renal abnormalities have been recognized.[106-109] A new treatment strategy aimed at enhancing urinary iron excretion could decrease systemic iron levels without these side effects. Reducing renal reabsorption of TBI and NTBI would be effective in reducing severe systemic iron levels since a decrease of circulating iron will induce iron release from tissue iron stores.[110] However, due to the redundancy in renal TBI and NTBI uptake mechanisms this approach may not be feasible. Moreover, excretion of high iron concentrations in urine may favor development of urinary tract infections[111] or iron-induced cytotoxicity in lower nephron segments, causing potential unfavorable complications.

Alternatively, our obtained insights in renal iron handling and subsequent toxicity may provide leads for the development of novel compounds intended to reduce the onset and/or progression of iron-mediated renal injury. Prevention of iron-mediated renal injury could be aimed at reduction of iron reabsorption, affecting intracellular pathways and/or inhibition of cell death processes (Figure 6.1). Inhibition of tubular iron reabsorption could reduce the enhanced labile iron levels within the tubular epithelial cells and subsequent

toxicity. However, it is required that iron transport inhibition includes PTs as well as lower nephron segments, in order to prevent iron accumulation along the entire nephron. As described above, inhibition of TBI and/or NTBI uptake may have the potential to reduce iron reabsorption but also has its challenges, such as iron transport redundancy. Therefore, future studies will need to determine whether individual or combined TBI and NTBI reabsorption inhibition therapy may have the potential to reduce accumulation of this labile iron species and consequent cytotoxic effects in renal tubular epithelial cells without increasing urinary bladder infections.

The deleterious effects of iron on renal tubular epithelial cells could be reduced by targeting intracellular pathways. Although this is presumed to be a complex interplay of multiple signaling and effector players, enhancing the cellular antioxidative response, potentially via Nrf2, or inhibiting inflammatory or ER stress cascades may be effective in reducing injury to renal tubular cells. Previously, the Nrf2 activator bardoxolone methyl has been successful in improving kidney function, decreasing the induction of renal injury and preventing the onset of end stage renal disease in clinical trials in patients with diabetes type 2 and CKD.[112-114] However, a phase 3 clinical trial including patients with diabetes type 2 and CKD stage 4 was terminated due to increased events of heart failure,[115] suggesting the degree of CKD progression may alter the effectiveness of this therapy. This shows that antioxidant induction therapies should be studied with caution to see in which CKD populations they may be used without causing serious adverse effects.

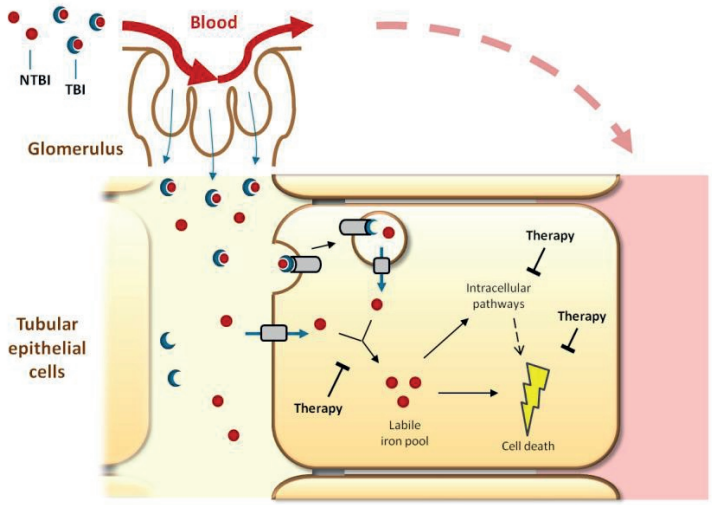


Figure 6.1: Treatment targets for prevention of iron-mediated renal tubular injury.
Prevention of iron-mediated renal injury in renal tubular epithelial cells could be aimed at 1) decreasing uptake of transferrin-bound iron (TBI) and/or non-transferrin-bound iron (NTBI); 2) affecting intracellular pathways such as oxidative stress, inflammation of endoplasmic reticulum (ER) stress; and 3) decreasing cell death processes (indicated with lightning sign) such as ferroptosis.

However, these strategies may have potential for reducing renal injury in CKD. Moreover, this therapy may also be effective in patients with systemic iron overload, where oxidative stress is also identified in the pathogenesis of iron-mediated renal injury (**Chapter 4**).[116,117]

Furthermore, iron-mediated renal injury could be reduced by preventing the terminal cell death processes induced by iron exposure. Iron exposure is shown to induce tubular atrophy and tubular necrosis. [117-119] As described above, these could be due to cell death processes of iron-dependent ferroptosis.[87] Evidence is growing that ferroptosis is involved in different AKI pathologies.[120] Interestingly, ferroptosis inhibitors are shown to attenuate morphological tubular injury following renal ischemia-reperfusion injury in mice.[121] It remains to be investigated whether ferroptosis is also involved in CKD and/or renal injury observed with systemic iron overload disorders.

In a broader perspective, it is conceivable that the exact molecular mechanisms of iron handling and subsequent injury to renal tubular cells vary for diseases of different etiology. Therefore, it will be essential to study the applicability of novel treatment modalities in detail for each of the different pathologies.

PERSPECTIVES FOR FUTURE RESEARCH

In this thesis we have demonstrated the presence of excess iron in human kidney and urine in relation to renal injury. Moreover, we have increased the insights into the mechanisms that are involved in renal iron handling processes and their potential subsequent toxicity. These results serve as the first steps towards future research into elucidating whether iron may initiate renal injury in systemic iron overload and aggravate the progression of renal injury in CKD.

In vitro and animal studies have examined associations between renal iron accumulation and injury in various disorders of CKD and systemic iron overload, but both are relatively unexplored in humans. Whereas animal studies have shown that iron accumulates in the kidney and urine in various CKD pathologies, longitudinal studies in patients with CKD are required to examine if disease progression results in a higher iron concentrations in the kidney or urine in humans. In addition, animal and subsequent human studies are warranted to assess whether renal iron deposition may be the cause of renal injury progression. This includes examination of molecular injury mechanisms that are involved in this process and interventions for renal iron reduction, such as an iron-restricted diet or administration of an iron chelator. In systemic iron overload, future studies should initially focus on increasing evidence on renal iron accumulation in these disease pathologies since the current evidence for iron accumulation in the kidney in animal models and patients with systemic iron overload is limited. Renal iron accumulation could be assessed by studying renal biopsies in systemic iron overload animal models and by making use of non-invasive T2* magnetic resonance imaging in

patients with systemic iron overload.[92,96] Subsequently, animal and human studies are needed to assess whether renal iron accumulation precedes renal injury, and whether amelioration of iron accumulation reduces renal complications in systemic iron overload. Within both CKD and systemic iron overload disorders, specific research interests could be included, such as studying the role of PTs and DTs separately, *i.e.* by comparing iron accumulation in these tubules in renal biopsies, or examining the effect of filtration of increased systemic iron levels. Furthermore, observational study designs in humans would allow to identify renal iron handling among multiple disorders of systemic iron overload or CKD. As such, individual pathologies could be pointed out in which iron may play a large role in causing renal injury and where therapies for reducing iron-mediated renal injury may be specifically beneficial for future patient care.

CONCLUSION

The results described in this thesis have expanded our knowledge into the mechanisms of renal iron handling in health, systemic iron overload and CKD. As such, we obtained a better understanding of the potential of iron to induce renal injury. Our results may aid in future studies aimed at decreasing iron-mediated injury in the kidney.

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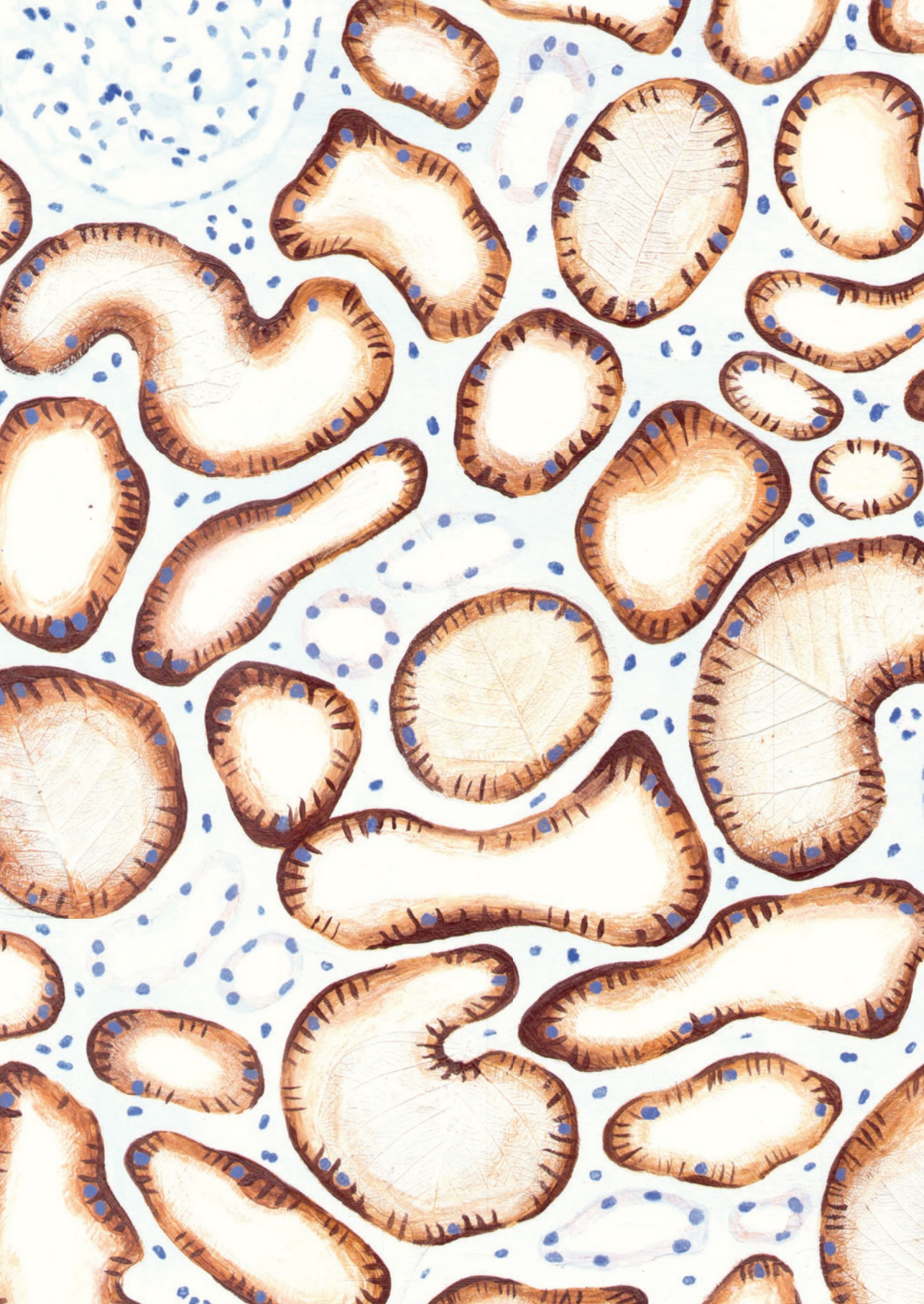


7

Summary
Samenvatting

SUMMARY

The metal iron (Fe) is essential for a large number of processes in the human body. However, it can also be toxic by catalyzing the formation of reactive oxygen radicals. Our current understanding of systemic and cellular iron homeostasis is mainly derived from studies in hepatocytes, erythrocyte precursors, enterocytes and macrophages. Knowledge of iron homeostasis in the kidney is limited, despite the presence of many proteins involved in cellular iron handling. The kidney is thought to play a crucial role in preservation of the body's iron, but the exact localization of several iron handling proteins in the various renal tubular segments in physiological conditions is still unclear. Moreover, due to the potential differential expression of iron handling proteins in the nephron segments, and the expression of addition kidney-specific iron handling proteins, renal iron handling may be different from iron handling processes observed in other organs.

The human body has limited physiological mechanisms to regulate iron excretion. As a consequence, disturbed iron acquisition, i.e. via the intestine in hereditary hemochromatosis or via additional frequent red blood cell transfusions in β-thalassemia syndromes, can result in systemic iron overload and, consequently, increase renal iron exposure. Evidence of renal iron deposition and renal dysfunction in patients with β-thalassemia syndromes supports an association between increased iron exposure and renal injury in conditions of systemic iron overload. Furthermore, in disorders of chronic kidney disease (CKD), renal iron deposition and increased urinary iron levels are described. Moreover, reduction of systemic iron levels has been reported to reduce renal iron accumulation and tubular injury in various CKD animal models, suggesting a relation may exist between renal iron accumulation and renal injury in CKD. In this thesis, we examined renal tubular iron handling in human health, systemic iron overload disorders and CKD. As such, we aimed to gain insights into the molecular mechanisms of renal tubular iron handling and the detrimental role of iron in renal injury.

Circulating iron can be filtered by the glomerulus, and is almost completely reabsorbed by the proximal and distal tubular epithelial cells (PTs and DTs, respectively), preventing excretion of this scarce metal in urine. Although increased urinary iron levels were found in various pathologies, in remains unclear whether urinary iron excretion may be caused by glomerular filtration of increased circulating iron levels and/or disturbed tubular iron reabsorption. In **Chapter 2**, we examined whether both processes could contribute to urinary iron excretion and renal injury. Therefore, we compared plasma and urine iron parameters and urinary tubular injury markers between patients with systemic iron overload, patients with renal tubular dysfunction and healthy controls. Expectedly, plasma iron parameters were increased only in patients with systemic iron overload, but urinary iron levels were enhanced in both patient groups compared to healthy controls. We demonstrated that glomerular filtration of increased circulating iron levels contributed to iron excretion in urine, since urinary iron levels associated with elevated plasma iron

parameters in systemic iron overload patients, as indicated by elevated transferrin saturation (TSAT), which is a marker for circulating iron loading. Moreover, in patients with β-thalassemia major, in which circulating iron parameters were severely elevated, iron levels in urine also associated with plasma ferritin levels, which are an indicator of renal tubular iron stores. Furthermore, disturbed tubular reabsorption contributed to urinary iron and transferrin excretion, since iron and transferrin levels in urine associated with urinary excretion of glutathione s-transferase-pi-1-1 (GSTP-1-1) in patients with tubular dysfunction, which is a marker for DT injury and, as such, confirms renal injury in these patients. Interestingly, filtration of high circulating iron levels also related to renal injury, since we found that levels of iron and transferrin in patients with systemic iron overload associated with urinary concentration of kidney injury marker 1 (KIM-1), a marker for injury for PTs.

In disorders of systemic iron overload, iron largely saturates the circulating iron binding protein transferrin (transferrin-bound iron, TBI) and also binds to low-molecular weight ligands, termed non-transferrin-bound iron (NTBI). In **Chapter 3**, we studied the molecular mechanisms of TBI and NTBI uptake in human conditionally immortalized PT epithelial cells (ciPTECs), focusing on divalent metal transporters ZIP8 and ZIP14. We first characterized iron handling in ciPTECs, by showing that both fluorescently labeled transferrin (as TBI) and ^{55}Fe (as NTBI) were internalized by ciPTECs. In addition, the iron export protein ferroportin was localized at the ciPTEC basolateral side and showed functional iron export characteristics, since small interfering RNA (siRNA) silencing increased cellular ^{55}Fe content compared to scrambled control, while ^{55}Fe in the exposure solution was decreased. When studying NTBI uptake, we found that both ZIP8 and ZIP14 localized to the ciPTEC plasma membrane, but ^{55}Fe uptake was not affected by siRNA silencing of either ZIP8 or ZIP14 alone. In contrast, simultaneous silencing of both transporters reduced ^{55}Fe uptake compared to control, indicating that both transporters are involved in NTBI uptake and show redundancy. Furthermore, transferrin receptor 1 (TfR1) and ZIP14, but not ZIP8, colocalized with early endosome antigen 1 (EEA1) staining. Both TfR1 and ZIP14 also colocalized with fluorescently labeled transferrin uptake. Moreover, ZIP14 silencing decreased ^{55}Fe uptake after ^{55}Fe-transferrin exposure, suggesting ZIP14 also mediates endosomal transport of TBI-derived iron towards the cytosol.

Reports of renal iron accumulation and renal injury in patients with systemic iron overload suggest that chronic exposure to increased iron levels results in nephrotoxicity. In acute iron exposure conditions, activation of the major cellular antioxidative pathway coordinated by Nuclear factor erythroid 2-related factor 2 (Nrf2) is known to protect from oxidative cellular injury. However, Nrf2 is also described to become exhausted in chronic renal stress. Therefore, **Chapter 4** examined the hypothesis that Nrf2 exhaustion as a result of iron-mediated oxidative stress underlies renal injury in chronic iron overload in ciPTECs. Chronic iron exposure induced iron accumulation, cytosolic reactive oxygen

species (ROS) formation and increased *heme oxygenase 1 (HMOX-1)* mRNA expression, a marker of cellular oxidative stress. Moreover, this was accompanied by nuclear translocation of Nrf2 and induction of its target NAD(P)H quinone dehydrogenase 1 (NQO1) on protein level. To simulate ciPTEC Nrf2 exhaustion we applied the Nrf2 inhibitor trigonelline. Indeed, addition of trigonelline to iron exposure decreased Nrf2 nuclear translocation and NQO1 protein levels. Interestingly, although iron and trigonelline incubated cells showed reduced cytosolic ROS levels, *HMOX-1* mRNA levels were still increased. Moreover, iron and trigonelline incubation induced *CHOP* mRNA expression, which is an indicator of endoplasmic reticulum stress, and ferritin protein levels. Although ferritin functions as intracellular iron storage molecule, it is also induced by other stress-related mechanisms such as inflammation. Altogether, although Nrf2 exhaustion did not enhance oxidative stress formation in ciPTECs, other stress-related mechanisms could induce PT cytotoxicity during high iron exposure.

In **Chapter 5**, we described our immunohistochemical studies into the presence and localization of iron handling proteins in renal biopsies of healthy controls and patients with various CKD pathologies. In these biopsies, we studied the expression of iron handling proteins in relation to iron deposition and renal injury. Iron was deposited in both PTs and DTs in 33% of the CKD biopsies, predominantly in pathologies with glomerular dysfunction, but was absent in controls. In the healthy kidney, we detected the iron uptake transporters ZIP8, ZIP14 and divalent metal transporter 1 (DMT1), iron storage proteins L- and H-ferritin and iron exporter ferroportin in PTs, indicating PTs are equipped for iron recycling. In contrast, DTs only expressed iron uptake transporters ZIP8, ZIP14 and DMT1. In the various CKD pathologies, iron accumulation associated with increased intensity of iron importers ZIP8 and ZIP14, storage proteins L- and H-ferritin, and/or decreased expression of iron exporter ferroportin, suggesting that iron loading in CKD may be the result of increased iron acquisition and/or decreased iron export. Iron deposition also associated with oxidative cellular injury, as indicated by enhanced renal heme oxygenase 1 (HO-1) protein staining, indicating that iron deposition in CKD may contribute to renal injury through oxidative stress.

Chapter 6 discusses the findings described in this thesis and gives implications and suggestions for future research into renal iron handling. The results of this thesis have expanded our knowledge into the mechanisms of renal iron handling in health, systemic iron overload and CKD. As such, we gained more insights in the potential of iron to induce renal injury, which can aid in decreasing iron-mediated injury in the kidney.

SAMENVATTING

Het metaal ijzer (Fe) is essentieel voor een groot aantal processen in het menselijk lichaam. Echter, ijzer kan ook schadelijk zijn omdat het de vorming van reactieve zuurstofradicalen kan katalyseren. Inzicht in hoe ons lichaam omgaat met ijzer komt grotendeels van studies in levercellen (hepatocyten), voorloper rode bloedcellen (erytrocyten), darmcellen (enterocyten) en ontstekingscellen (macrofagen). We weten echter nog weinig van het ijzer metabolisme in de nier. De nier lijkt een cruciale rol te spelen bij het in stand houden van de hoeveelheid ijzer in het lichaam. Ondanks dat de aanwezigheid van vele eiwitten die betrokken zijn bij moleculaire en cellulaire ijzer processen in de nier zijn aangetoond, is de precieze lokalisatie van deze eiwitten in de nier onder normale omstandigheden nog niet opgehelderd. Bovendien lijkt de manier waarop de nier omgaat met ijzer anders te zijn dan deze processen in andere organen, door verschillen in de aanwezigheid van eiwitten die betrokken zijn bij ijzertransport of -stapeling. Daarbij spelen de complexe organisatie van de nier en de aanwezigheid van nierspecifieke ijzer-gerelateerde eiwitten ook een rol.

Het menselijk lichaam neemt ijzer op vanuit de voeding in de darm. Echter, het lichaam heeft maar beperkte mogelijkheden om de uitscheiding van ijzer actief te reguleren. Dit heeft tot gevolg dat een te hoge ijzeropname in de darm kan leiden tot systemische ijzerstapeling, zoals bij de aangeboren aandoening hemochromatose. Ook frequente bloedtransfusies, die gegeven worden als behandeling van β-thalassemie syndromen, kunnen leiden tot te veel ijzer in het lichaam. In deze ziektebeelden wordt de nier blootgesteld aan verhoogde ijzerconcentraties. Bevindingen van ijzerstapeling in de nier en verstoorde nierfunctie in patiënten met β-thalassemie syndromen ondersteunen de gedachte dat verhoogde ijzerblootstelling in de nier door systemische ijzerstapeling kan leiden tot nierschade, waaronder ook in de nierbuisjes. Een relatie tussen ijzerstapeling en schade in de nier wordt ook gesuggereerd door bevindingen in patiënten met chronische nierziekten. De relatie tussen ijzerstapeling in de nier en nierschade is verder aangetoond in diermodellen van chronische nierziekten, waarbij het verlagen van de systemische ijzerconcentratie leidde tot een vermindering van ijzerstapeling en schade in de nier. In dit proefschrift zijn de processen van ijzermetabolisme in de gezonde nier in de mens nader onderzocht, alsmede in condities van systemische ijzerstapeling en chronische nierziekten. Op deze manier hebben we meer inzicht gekregen in de moleculaire mechanismen van ijzertransportprocessen in de nier en de mogelijke rol van ijzer bij het ontstaan van nierschade.

IJzer dat aanwezig is in de bloedbaan kan door de glomerulus van de nier worden gefilterd waarna het in het lumen van de nierbuisjes terechtkomt. Vervolgens wordt al het ijzer opgenomen door cellen van de proximale (PTs) en distale nierbuisjes (DTs) om

vervolgens getransporteerd te kunnen worden naar het bloed. Zo wordt voorkomen dat het lichaam dit schaarse metaal met de urine verliest. Bij verschillende ziektebeelden zijn verhoogde ijzerconcentraties in de urine gevonden, maar of dit komt door filtratie van verhoogde ijzerconcentraties in het bloed door de glomerulus en/of door verminderde opname door de nierbuisjes was nog onbekend. In **Hoofdstuk 2** hebben we gekeken of beide processen kunnen bijdragen aan de uitscheiding van ijzer in de urine en aan het ontstaan van nierschade. Hiervoor hebben we de ijzerconcentraties in bloed en urine, maar ook markers voor nierschade in urine, vergeleken tussen patiënten met systemische ijzerstapeling, patiënten met verstoorde nierbuisfunctie en gezonde vrijwilligers. Zoals verwacht was de ijzerconcentratie in het bloed alleen verhoogd in patiënten met systemische ijzerstapeling; de ijzerconcentratie in de urine daarentegen was in beide patiëntengroepen verhoogd in vergelijking met gezonde vrijwilligers. We hebben laten zien dat glomerulaire filtratie van verhoogde ijzerconcentraties in het bloed bijdraagt aan de uitscheiding van ijzer in de urine, omdat de hoge ijzerspiegels in de urine geassocieerd waren met verhoogde ijzerconcentraties in het bloed in patiënten met systemische ijzerstapeling. Patiënten met een verstoorde nierbuisfunctie hadden verhoogde concentraties van glutathion s-transferase-pi-1-1 (GSTP-1-1) in de urine, een marker voor schade aan cellen van de DT. In deze patiënten waren deze concentraties geassocieerd met de hoge concentraties aan ijzer en transferrine in de urine. Bovendien waren ijzer en transferrine spiegels in urine van patiënten met systemische ijzerstapeling geassocieerd met de aanwezigheid van de nierschademarker 1 (KIM-1) in urine. Dit duidt op schade aan de cellen van de PTs. Deze resultaten laten zien dat zowel filtratie van verhoogde ijzerconcentraties in het bloed door de glomerulus als verminderde ijzeropname door de nierbuisjes kunnen bijdragen aan de uitscheiding van ijzer in de urine en nierschade.

Bij systemische ijzerstapeling is het ijzertransporteiwit transferrine grotendeels verzadigd met ijzer (genaamd transferrine-gebonden ijzer, TBI) en bindt ijzer vervolgens ook aan andere moleculen. Dit wordt niet-transferrine-gebonden ijzer (NTBI) genoemd. Omdat de binding van ijzer aan deze andere moleculen mogelijk minder stabiel is dan in het geval van TBI, kan NTBI een schadelijk vorm van ijzer zijn. In **Hoofdstuk 3** hebben we de moleculaire mechanismen van de opname van TBI en NTBI in humane conditioneel-geïmmortaliseerde PT epitheel cellen (ciPTECs) bestudeerd. Hierbij hebben we specifiek gekeken naar de divalent-metaaltransporters ZIP8 en ZIP14. De ciPTECs bleken in staat het ijzer te metaboliseren, want deze cellen konden zowel het fluorescerend-gelabelde transferrine (als maat voor TBI) als het radioactief-gelabelde ijzer (^{55}Fe, als NTBI) opnemen. Bovendien was het ijzerexporteiwit ferroportin aanwezig in de basaalmembraan van de ciPTECs (de zijde die in contact staat met het bloed). Ferroportin functioneerde als ijzerexporteiwit, aangezien een verlaging van de ferroportinconcentratie door genblokkade leidde tot een toegenomen hoeveelheid ^{55}Fe in de cellen, terwijl de hoeveelheid ^{55}Fe in het celkweekmedium was verminderd. Vervolgens hebben we NTBI opname bestudeerd. We konden de aanwezigheid van ZIP8 en ZIP14 op het ciPTEC-

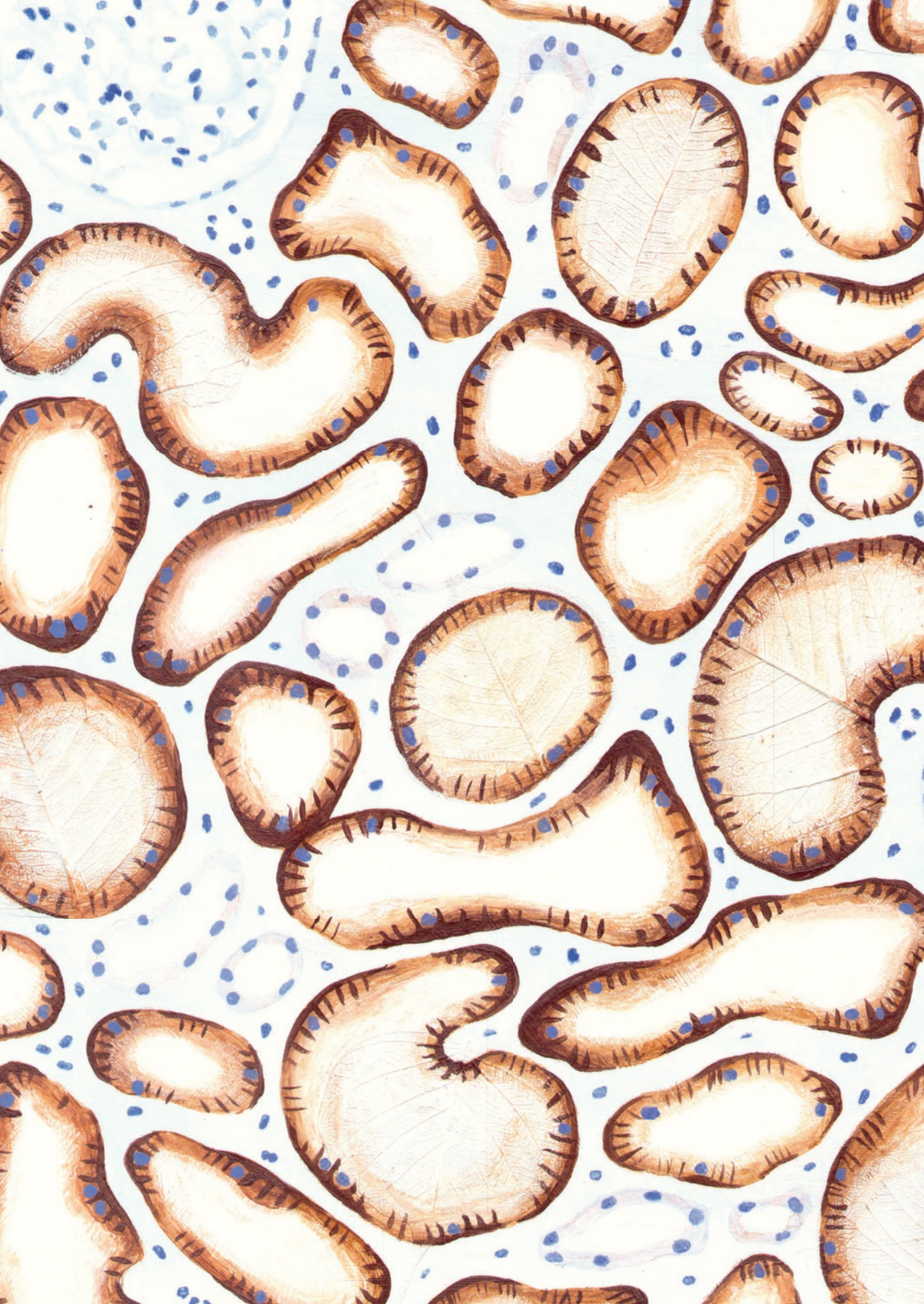
plasmamembraan aantonen. Echter leidde verlaging van de concentratie van ZIP8 of ZIP14 afzonderlijk niet tot een verandering van de ^{55}Fe concentratie in de cellen. Verlaging van beide transporters tegelijk leidde wel tot een afname van de concentratie ^{55}Fe in de cellen. Dit betekent dat beide transporters betrokken zijn bij de opname van NTBI en elkaars functie (gedeeltelijk) kunnen overnemen. Tenslotte hebben we de opname van TBI bestudeerd. Het is bekend dat TBI door de niercel wordt opgenomen middels endocytose door transferrine receptor 1 (TfR1). De lokalisatie van de transporters TfR1 en ZIP14, maar niet ZIP8, kwam overeen met die van het endosoom antigeen 1 (EEA1), wat suggereert dat TfR1 en ZIP14 waarschijnlijk betrokken zijn bij de opname van TBI. Bovendien viel de aanwezigheid van zowel TfR1 als ZIP14 samen met de endosomen waarin fluorescerend-gelabelde transferrine werd opgenomen. Ook vonden we dat een verlaagde concentratie van ZIP14 leidde tot een afname in ^{55}Fe opname na blootstelling aan ^{55}Fe-transferrine. Dit betekent dat ZIP14 betrokken is bij de opname van ijzer afkomstig van TBI vanuit het endosoom.

In patiënten met systemische ijzerstapeling is ijzerstapeling in en schade aan de nier gevonden. Dit wekt de suggestie dat chronische ijzerblootstelling kan leiden tot niertoxiciteit. Dit wordt mogelijk veroorzaakt door schadelijke effecten van reactieve zuurstofradicalen, een proces wat ook wel oxidatieve stress wordt genoemd. Tijdens acute ijzerblootstelling beschermt de zogenaamde 'antioxidant response' de cel tegen de schadelijke effecten van oxidatieve stress. Dit wordt gecoördineerd door de transcriptiefactor Nucleaire factor erythroid 2-gerelateerde factor 2 (Nrf2). Echter, het is bekend dat in condities van chronische stress de hoeveelheid Nrf2 in de nier uitgeput kan raken. Daarom hebben we in **Hoofdstuk 4** bestudeerd of nierschade in ciPTECs bij chronische ijzerblootstelling kan ontstaan door Nrf2 uitputting als gevolg van ijzer-gemedieerde oxidatieve stress. Chronische ijzerblootstelling leidde tot ijzerstapeling in de cel, productie van reactieve zuurstofradicalen en verhoogde aanwezigheid van het *heem oxygenase 1*-gen (*HMOX-1*), wat een marker is voor oxidatieve stress in de cel. De ijzerblootstelling activeerde het eerder beschreven Nrf2 beschermingsmechanisme. We vonden namelijk verhoogde concentraties van Nrf2 in de celkern en een toename van het eiwit NAD(P)H chinon dehydrogenase 1 (NQO1), welke gestimuleerd wordt door Nrf2. Om Nrf2 uitputting in ciPTECs na te bootsen hebben we de Nrf2 remmer trigonelline gebruikt. Toevoeging van trigonelline aan de ijzerblootstelling verminderde de Nrf2 concentraties in de celkern en de hoeveelheid NQO1 in de cel. In tegenstelling tot wat we verwacht hadden, leidde Nrf2 remming tot een afname van de hoeveelheid van reactieve zuurstofradicalen in de cel, maar was de *HMOX-1* genexpressie nog steeds verhoogd. Blootstelling aan trigonelline en ijzer leidde tevens tot een toegenomen aanwezigheid van het gen *CHOP*, wat wijst op endoplasmatisch-reticulum stress. Daarbij was ook het eiwit ferritine verhoogd, welke een afspiegeling is van de hoeveelheid ijzer die opgeslagen is in de cel, maar ook kan worden verrijkt door andere stress-gerelateerde mechanismen. Samengevat, ondanks dat Nrf2 remming geen toename van oxidatieve stress in ciPTECs

veroorzaakt leidt dit wel tot andere stress-gerelateerde mechanismen die leiden tot een toegenomen cytotoxiciteit in PTs tijdens langdurige blootstelling aan hoge ijzerconcentraties.

Hoofdstuk 5 beschrijft immunohistochemische studies omtrent de aanwezigheid en lokalisatie van verschillende eiwitten die betrokken zijn bij het ijzermetabolisme in nierbiopten van gezonde vrijwilligers en van patiënten met verschillende vormen van chronische nierziekten. Bovendien hebben we de aanwezigheid van deze eiwitten in relatie tot ijzerstapeling en nierschade in deze biopten bestudeerd. In 33% van de onderzochte biopten kwam ijzerstapeling voor in cellen van zowel PTs als DTs. Dit bleek vooral bij aandoeningen met schade aan de glomerulus. In de gezonde nier kon de aanwezigheid van de ijzeropnametransporters ZIP8, ZIP14 en divalent-metaaltransporter 1 (DMT1), ijzeropslageiwitten L- en H-ferritine en de ijzerexporteur ferroportin in cellen van de PTs worden aangetoond. Dit betekent dat PTs zijn uitgerust om ijzer te recyclen vanuit het nierbuisje terug naar de bloedbaan. Daarentegen konden we in DTs alleen de ijzeropnametransporters ZIP8, ZIP14 en DMT1 detecteren. In biopten van verschillende chronische nierziekten bleek ijzerstapeling geassocieerd te zijn met een toegenomen aanwezigheid van de ijzerimporteurs ZIP8 en ZIP14 en de ijzeropslageiwitten L- en H-ferritine, en/of afgenomen hoeveelheid van de ijzerexporteur ferroportin. Dit betekent dat ijzerstapeling in patiënten met chronische nierziekten veroorzaakt kan worden door een toegenomen ijzeropname en/of verminderde ijzerexport in de niercellen. IJzerstapeling liet ook een relatie zien met oxidatieve stress in de niercellen, aangezien het heem oxygenase 1 (HO-1) eiwit verhoogd aanwezig was in de nierbiopten. Dit betekent dat ijzerstapeling tijdens chronische nierziekten oxidatieve stress kan veroorzaken wat resulteert in nierschade.

In **Hoofdstuk 6** worden de bevindingen van dit proefschrift bediscussieerd en tevens worden suggesties gedaan voor toekomstig onderzoek naar het ijzermetabolisme in de nier. Samenvattend hebben de bevindingen uit dit proefschrift onze kennis van de moleculaire mechanismen van het ijzermetabolisme in de nier van de gezonde mens en tijdens systemische ijzerstapeling of chronische nierziekten vergroot. Bovendien hebben we meer inzichten gekregen in de potentiële rol van ijzer in het veroorzaken en/of verergeren van nierschade. Mogelijk kunnen deze inzichten in de toekomst bijdragen aan het ontwikkelen van nieuwe therapieën om ijzer-gemedieerde schade in de nier te verminderen.



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List of publications

List of abbreviations

Research data management

Curriculum Vitae

PhD portfolio

LIST OF PUBLICATIONS

Van Raaij SEG, Rennings AJ, Biemond BJ, Schols SEM, Wiegerinck ETG, Roelofs HMJ, Hoorn EJ, Walsh SB, Nijenhuis T, Swinkels DW, van Swelm RPL. Iron handling by the human kidney: Glomerular filtration and tubular reabsorption both contribute to urinary iron excretion. *American Journal of Physiology – Renal Physiology, accepted for publication*

Van Raaij SEG, Srai SKS, Swinkels DW, van Swelm RPL. Iron uptake by ZIP8 and ZIP14 in human proximal tubular epithelial cells. *BioMetals, accepted for publication*

Van Raaij SEG, Masereeuw R, Swinkels DW, van Swelm RPL. Inhibition of Nrf2 alters cell stress induced by chronic iron exposure in human proximal tubular epithelial cells. Toxicology Letters 2018; 295: 179-186.

Van Raaij S, van Swelm R, Bouman K, Cliteur M, van den Heuvel MC, Pertijs J, Patel D, Bass P, van Goor H, Unwin R, Srai SK, Swinkels D. Tubular iron deposition and iron handling proteins in human healthy kidney and chronic kidney disease. Scientific Reports 2018; 8(1): 9353. Erratum in: Scientific Reports 2018; 8(1): 13390.

ABBREVIATIONS

546-Tf	Alexa546-labeled transferrin
Apotransferrin	Iron deficient transferrin
CHOP	CCAAT-enhancer-binding protein homologous protein
CiPTEC	Conditionally immortalized proximal tubular epithelial cell
CKD	Chronic kidney disease
CM-H_2DCFDA	2', 7'-dichlorodihydrofluorescein di-acetate
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DMT1	Divalent metal transporter 1
DFX	Deferasirox
DT	Distal tubule
DTEC	Distal tubular epithelial cell
DN	Diabetic nephropathy
DNA	Advanced diabetic nephropathy
DNE	Established diabetic nephropathy
DFX	Deferasirox
EEA1	Early endosome antigen 1
eGFR	Estimated glomerular filtration rate
ER	Endoplasmic reticulum
FCS	Fetal calf serum
Fe	Iron
Fe-NTA	Ferric nitrilotriacetate
Ferric citrate	FeC
Ferric iron	Fe^{3+}
Ferrous iron	Fe^{2+}
FPN1	Ferroportin
FSGS	Focal segmental glomerulosclerosis
FTH	Ferritin heavy chain (H-ferritin)
FTL	Ferritin light chain (L-ferritin)
GBM	Glomerular basement membrane
GCLM	Glutamate-cysteine ligase modifier subunit
GFR	Glomerular filtration rate
GSTP1-1	Glutathione s-transferase-pi-1-1
Grz	Glutathione reductase

HFE	Human hemochromatosis protein
HFE-HH	HFE-hereditary hemochromatosis
HH	Hereditary hemochromatosis
HMOX-1	Heme oxygenase 1 (mRNA)
HO-1	Heme oxygenase 1 (protein)
Holo-Tf	Holo-transferrin
Holotransferrin	Diferric transferrin
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
ICP-MS	Inductively coupled plasma mass spectrometry
IgAN	IgA nephropathy
IRE-IRP	Iron responsive element – Iron responsive protein
IQR	Interquartile range
LPI	Labile plasma iron
LN	Lupus nephritis
LTCC	L-type voltage-dependent calcium channel
KEAP1	Kelch like ECH associated protein 1
KH-H	Krebs-Henseleit HEPES buffer
KIM-1	Kidney injury molecule 1
MDA	Malondialdehyde
MDRD	Modifications of Diet in Renal Disease
Monotransferrin	Ferric transferrin
NAG	N-acetyl-β-D-glucosaminidase
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cell
NGALR	NGAL receptor
NQO1	NAD(P)H quinone dehydrogenase 1
Nrf2	Nuclear factor-erythroid 2-related factor 2
NTBI	Non-transferrin-bound iron
PAS	Periodic acid-Schiff
PCBP	Poly(rC)-binding protein
PT	Proximal tubule
PTEC	Proximal tubular epithelial cell
ROS	Reactive oxygen species
siRNA	Small interfering RNA
SEM	Standard error of the mean
STEAP3	Six-transmembrane epithelial antigen of prostate 3

TBA	Thiobarbituric acid
TBARs	Thiobarbituric acid-reactive substances
TBI	Transferrin-bound iron
TBMD	Thin basement membrane disease
TIBC	Total iron binding capacity
Tf	Transferrin
TfR1	Transferrin receptor 1
Trig	Trigonelline
TRPV5	Transient receptor potential cation channel subfamily V member 5
TSAT	Transferrin saturation
TTCC	T-type voltage-dependent calcium channel
TXNRD1	Thioredoxin reductase 1
UPR	Unfolded protein response

RESEARCH DATA MANAGEMENT

Appropriate research data management is important for safeguarding scientific integrity, open science, safekeeping of valuable datasets and the reuse of data. Research data presented in this thesis and obtained during this PhD trajectory at the Translational Metabolic Laboratory, department of Laboratory Medicine, at the Radboud university medical center (Radboudumc) were archived according to the Findable, Accessible, Interoperable and Reusable (FAIR) principles (Wilkinson *et al.* 2016, Scientific Data). Initially, raw and processed data was stored digitally on a local server of the department of Translational Metabolic Laboratory and on paper in the form of labjournals. Later, Labguru, a digital lab book, was introduced at this department that replaced the regular paper labjournals. Both the local server at the department and Labguru are supported by the Information and Communications Technology (ICT) of the Radboudumc. Labguru is daily backed up on the local server of the Radboudumc, while data stored on the local servers were replicated daily to servers of the Radboud University. The data files on Labguru and the local server are accessible by the associated scientific staff members. However, once the data are locked, the staff members can only view data files of the archive from Labguru and local servers, but not edit or remove any archived files. Studies in **Chapter 3** and **Chapter 4** used the patented cell line ciPTEC, which was developed from kidney tissue from an anonymous donor. Informed consent for use of this material was obtained. Human studies in **Chapter 2** and **Chapter 5** were conducted according to Dutch ethical guidelines and the principles of the Declaration of Helsinki. All participants gave written informed consent to participate in these studies, except when anonymized material left over from diagnostic care was used. In this case, in line with Dutch ethical guidelines, this material can be used without informed consent and approval from an ethical committee. The study protocol used in **Chapter 2** was approved by the Medical Ethical Review Committee and the Board of Directors of the Radboudumc. All data generated or analyzed in this thesis are included in published articles and its additional files are available from the associated corresponding authors on request.

CURRICULUM VITAE

Sanne Elisabeth Gerritje van Raaij werd geboren op 4 februari 1990 te Nijmegen. In 2008 behaalde zij haar VWO-diploma aan het Stedelijk Gymnasium Nijmegen. In datzelfde jaar begon ze aan de opleiding Biomedische Wetenschappen aan de Radboud Universiteit in Nijmegen. Ze deed haar bachelorstage bij de afdeling Fysiologie van het Radboudumc in het toenmalige Nijmegen Center for Molecular Life Sciences (NCMLS), onder leiding van Prof. dr. Joost Hoenderop, waar zij onderzoek deed naar de rol van S100 eiwitten in het reguleren van calcium kanaal TRPV5 in de nier. Ook was Sanne betrokken bij het curriculum Biomedische Wetenschappen als studentlid van de opleidingscommissie (OLC) Biomedische Wetenschappen en het Onderwijs Management Team-1 (OMT-1). Ze vervolgde haar opleiding met de master Biomedical Sciences aan de Radboud Universiteit en richtte zich op de hoofdvakken Toxicology en Human Pathobiology. Haar eerste masterstage deed Sanne op de afdeling Women's and Children's Health van het Karolinksa Institutet in Stockholm in Zweden. Onder leiding van dr. Lena Sahlin bestudeerde ze de rol van de hormoonverstorende stof bisfenol A op oestrogeenreceptoren in de baarmoeder van de rat. Tijdens haar tweede masterstage, onder leiding van Prof. dr. Roos Masereeuw, op de afdeling Farmacologie-Toxicologie van het Radboudumc in het NCMLS, ontwikkelde Sanne een nieuw kweekmodel van de muizentestis waarin ze de rol van bisfenol A en transporteiwitten tijdens ontwikkeling van het weefsel onderzocht. Sanne rondde haar masteropleiding *cum laude* af, en ontving daarnaast de Universitaire Studieprijs van de Radboud Universiteit en de 2014 *best Master thesis* prijs van de Nederlandse Vereniging voor Farmacologie.

In februari 2014 startte Sanne haar promotieonderzoek bij het Translationeel Metabool Laboratorium, onderdeel van de afdeling Laboratorium Geneeskunde, van het Radboudumc in het Radboud Institute for Molecular Life Sciences (RIMLS) in Nijmegen. Onder leiding van Prof. dr. Dorine Swinkels, Prof. dr. Roos Masereeuw en dr. Rachel van Swelm verrichte ze onderzoek naar de processen van het ijzermetabolisme in de gezonde nier en in condities van systemische ijzerstapeling en chronische nierziekten. Tijdens haar onderzoek heeft Sanne zeven studenten begeleid en haar werk gepresenteerd op verschillende internationale congressen. Haar onderzoek heeft geleid tot meerdere artikelen die gepubliceerd zijn in wetenschappelijke tijdschriften en dit proefschrift. Gedurende haar promotietraject rondde ze tevens de postdoctorale opleiding tot toxicoloog en de Basiscursus Regelgeving en Organisatie voor Klinische Onderzoekers (BROK) succesvol af. Sanne is momenteel werkzaam als Country Approval Associate bij de contract research organisatie PPD in Bennekom en ondersteunt hier klinisch onderzoek.

PhD PORTFOLIO

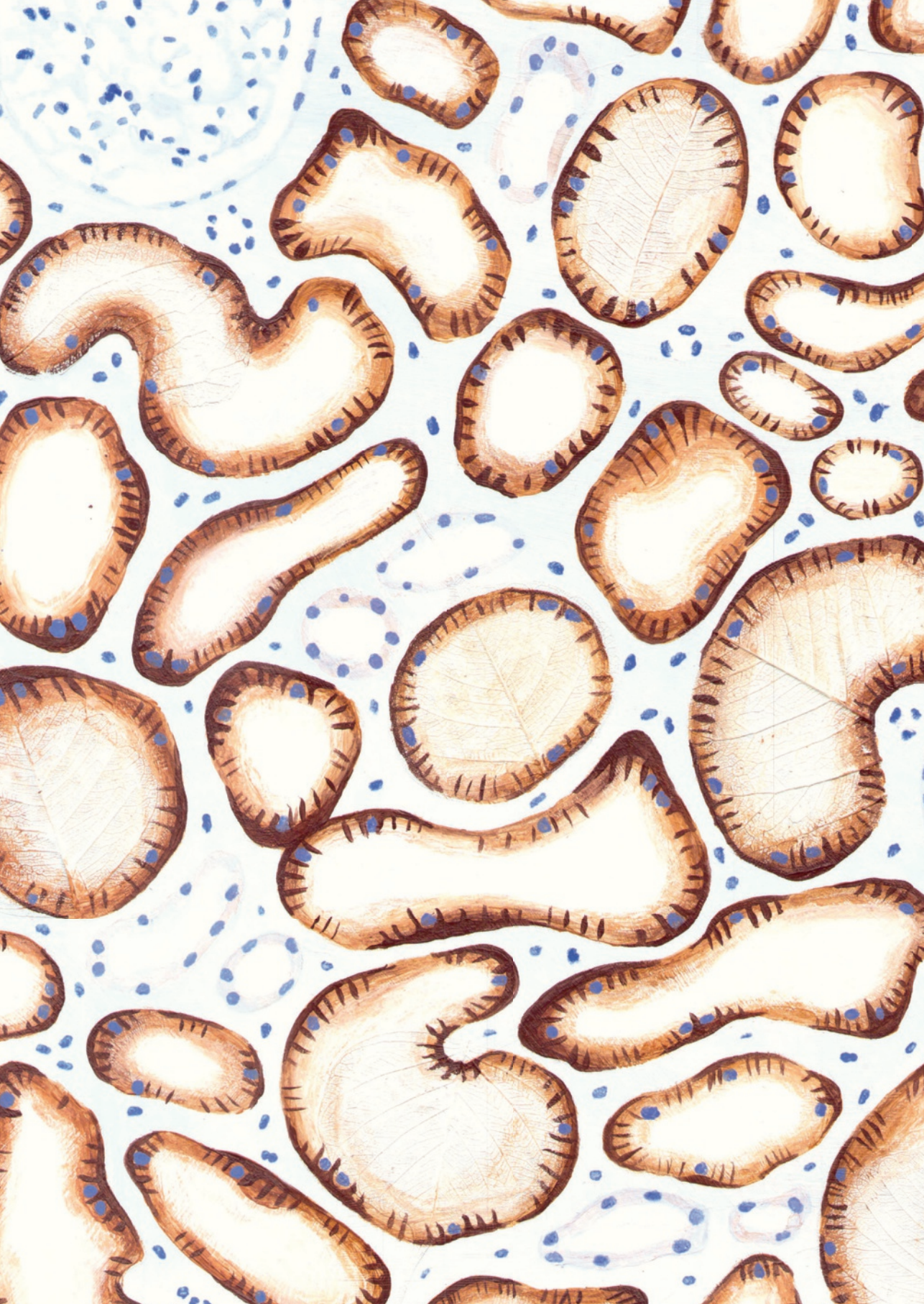
Institute for Molecular Life Sciences
Radboudumc

Name PhD student:	***SEG van Raaij***	**PhD period:**	***01-02-2014 – 31-01-2018***
Department:	***Translational Metabolic Laboratory***	**Promotors:**	***Prof.dr. D.W. Swinkels*** ***Prof. Dr. R. Masereeuw***
Graduate school:	***Radboud Institute for Molecular Life Sciences***	**Co-promotor:**	***Dr. R.P.L. van Swelm***

TRAINING ACTIVITIES	**Year(s)**	**ECTS**
a) Courses & Workshops		
- Introduction day Radboudumc	2014	0.5
- RIMLS Graduation Course	2014	2.0
- Course 'Management voor promovendi'	2015	3.0
- Course 'Scientific Integrity'	2015	1.0
- PET course 'Risk assessment'	2015	1.5
- PET course 'Ecotoxicology'	2016	3.0
- PET course 'Epidemiology'	2016	1.5
- BROK course, exam and registration	2016	1.5
b) Seminars & Lectures		
- RIMLS Seminars and Technical forum	2014-2017	1.9
- RIMLS Radboud Research Rounds (Plus)	2014-2017	1.1
- Kidney research theme lunch meeting*	2015-2018	1.65
- TML research meeting***	2015-2018	2.95
c) (Inter)national Symposia & Congresses		
- 2nd Benelux congress on Physiology and Pharmacology	2014	0.25
- European Iron Club Verona#	2014	1.5
- RIMLS PhD retreat##*	2014, 2015, 2016, 2017	2.75
- RIMLS New Frontiers	2014, 2015	2.0
- Seventh Congress of the International BioIron Society**	2017	2.25

TEACHING ACTIVITIES	**Year(s)**	**ECTS**
d) Supervision of internships		
- Master student A. Sebregts	2014-2015	2.5
- Master student L. Miesen	2014-2015	2.5
- Honours bachelor student K. Bouman	2015-2016	0.8
- Master student C. van der Horst	2016-2017	2.0
- Master student M. Platenburg	2017	2.0
- Bachelor student A. Geerlings	2017	1.0
- Master student M. Poppa	2017-2018	1.7
TOTAL		**42.85**

Oral and poster presentations are indicated with a * and # after the name of the activity, respectively.



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Dankwoord | Acknowledgements

DANKWOORD

Het is echt zover; mijn proefschrift is af. Ik sluit hiermee een periode van 5 jaar af, waarin ik veel heb geleerd over onderzoek doen, de wetenschap en mezelf. Maar ik had alle voorbereidingen voor dit proefschrift nooit kunnen volbrengen als ik bij de 'downs' niet had kunnen bouwen op heel veel mensen om mij heen. Maar ook tijdens de 'ups', want ik heb de mooie momenten en mijlpalen mogen vieren met velen.

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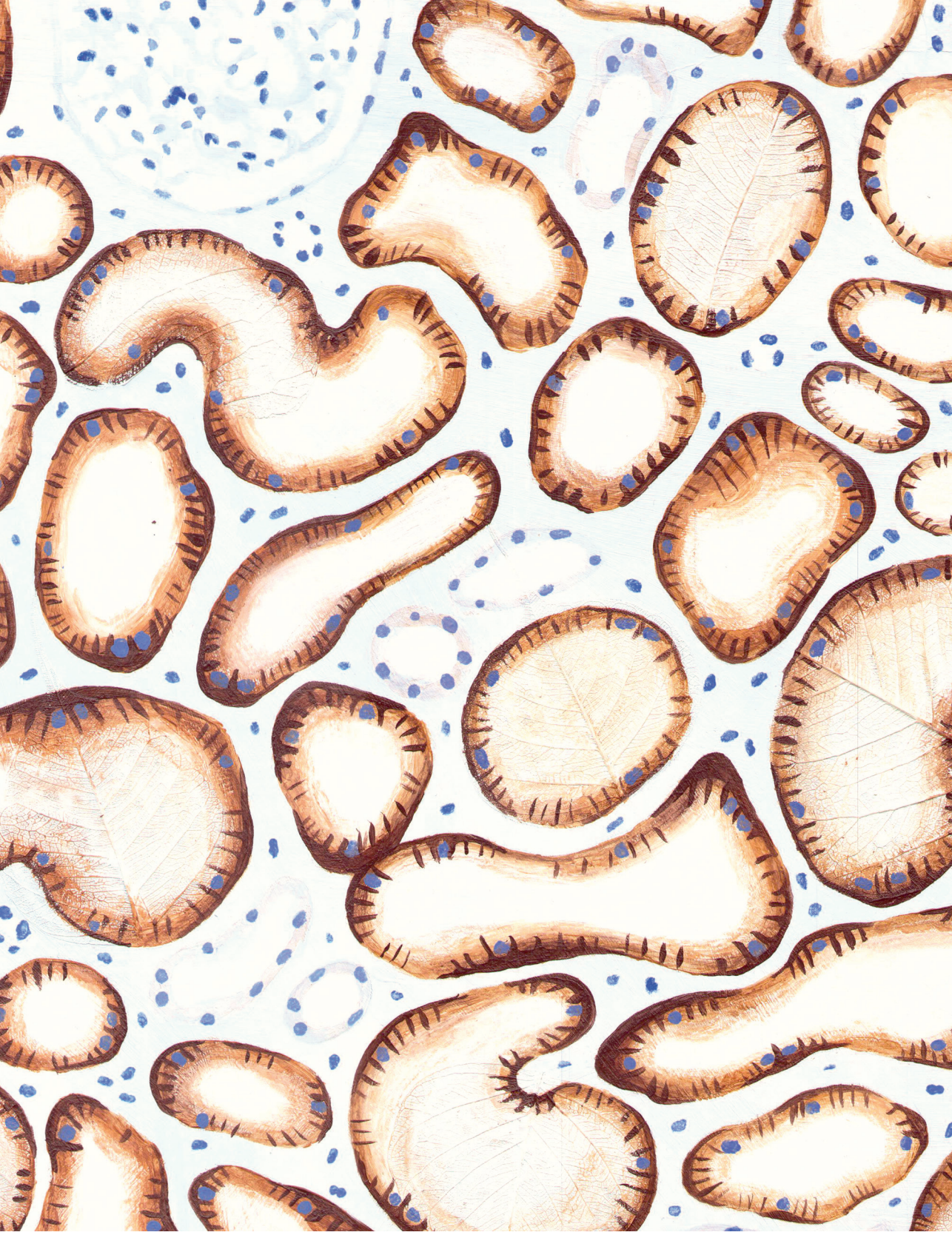
Liefs, Sanne



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